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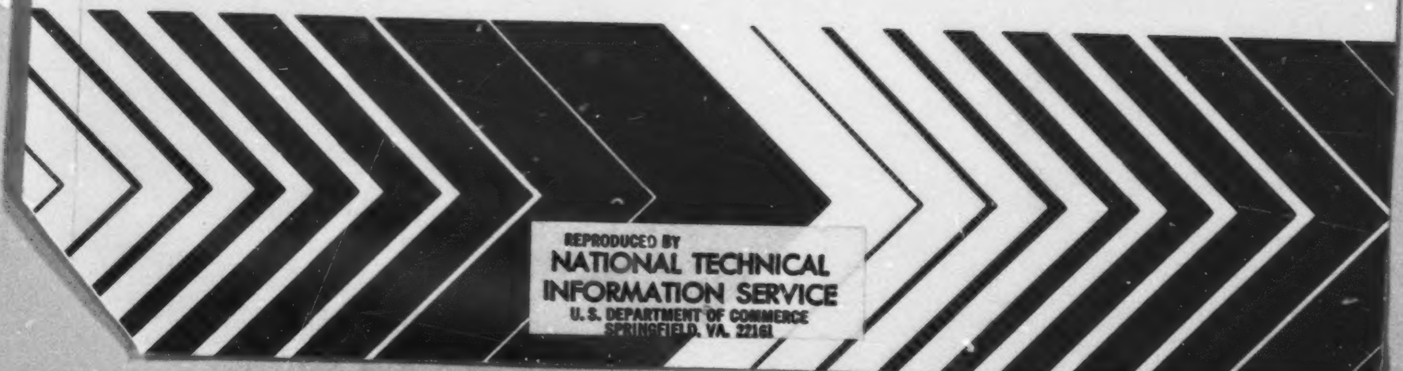

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This report describes studies wherein guinea pigs and mice were exposed to low levels of nitrogen dioxide either continuously or on an intermittent schedule. The major objective was to develop highly sensitive discriminants involving correlations of structural and functional alterations. The two major test areas developed were the quantitative measurements of Type 2 cell populations and protein leakage within the lung. The increase in number and size of Type 2 pneumocytes with NO_2 exposure is believed to represent a corresponding loss of Type 1 lung cells, and the protein leakage within the lung is in accord with the concept that the Type 1 cell is a critical barrier for fluid transport in the alveolar area. The authors suggest that their research data supports the hypothesis that to properly evaluate the possible health effects from air pollutants one must give attention to the subclinical cellular alterations that deplete the functional and structural reserves of organs which are the primary target for air pollutants.

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A BIOLOGIC INDICATOR FOR AIR POLLUTION

by

Russell P. Sherwin
University of Southern California Medical Center
Los Angeles, California 90033

Grants No. R800881 and R804545

Project Officer

Donald E. Gardner
Biomedical Research Branch
Health Effects Research Laboratory
Research Triangle Park, NC 27711

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NC 27711

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The report describes studies wherein guinea pigs and mice were exposed to low levels of nitrogen dioxide either continuously or on sensitive discriminants involving correlations of structural and functional alterations. The two major test areas developed were the quantitative measurements of Type 2 cell populations and protein leakage within the lung. The increase in number and size of Type 2 pneumocytes with NO_2 exposure is believed to represent a corresponding loss of Type 1 lung cells, and the protein leakage within the lung is in accord with the concept that the Type 1 cell is a critical barrier for fluid transport in the alveolar area. The authors suggest that their research data supports the hypothesis that to properly evaluate the possible health effects from air pollutants one must give attention to the subclinical cellular alterations that deplete the functional and structural reserves of organs which are the primary target for air pollutants.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

Studies with nitrogen dioxide (NO_2) were carried out at levels from 2 ppm to a low of 0.4 ppm, with either continuous or intermittent exposures and with the use of guinea pigs and mice. The major overall goal was to develop highly sensitive discriminants involving correlations of structural and functional alterations. Two major testing areas were developed, quantitative measurements of Type 2 cell populations and protein leakage in the lung. A series of four image analyzer studies showed that exposure of guinea pigs to continuous 2 ppm NO_2 resulted in an increased number of Type 2 pneumocytes ($p < .05$), that there was a very high correlation between hand and automated cell counts ($r > 7.7$; $p < .001$), there was high correlation between hand counts of alveoli and automated measurements of alveolar wall area ($r > .056$; $p < .002$), that duration of exposure had a significant effect ($F = 5.33$; $p < .05$), and that the Type 2 cell increased in size ($p < .025$), with duration of the exposure also significant ($p < .05$).

With respect to protein leakage, polyacrylamide gel electrophoresis (PAGE) quantitative measurements showed a greater protein content in the lung lavage fluid of guinea pigs that had been exposed to continuous 0.4 ppm NO_2 for 1 week ($p < .001$) as opoosed to a control group. In two studies of the urine of guinea pigs, the protein content was shown by PAGE and Lowry protein tests to be higher for the exposed group of animals ($p < .01$ and $p < .05$ respectively for those exposed to continuous and intermittent (4 hours per day) NO_2. A newly innovated test for protein content of lungs of mice, using a fluorescent protein label (fluorescamine) showed higher levels of protein in the lungs of mice exposed continuously to 0.47 ppm NO_2 for 12 ± 2 days ($p < .025$). The latter studies were expanded through the use of a molecular probe, horseradish peroxidase (HRP) as a means of quantitating plasma and other protein substances in lung cells, tissues, air spaces, and lymphatic channels. In three independent experiments, using continuous 0.6 ppm NO_2, intermittent 0.8 ppm NO_2, and intermittent 0.6 ppm NO_2, an increased HRP content of the lungs of the exposed animals was found (first two experiment) after three weeks of exposure $p < .05$ in each case. With respect to the third experiment, an elevation of HRP in the lungs of exposed animals occurred at 6 weeks ($p < .025$). Of the kidney HRP content studies, there was one significant difference ($p < .05$), again for an exposed group of animals. Parallel enzyme kinetic assays of HRP were also carried out for some of the studies and confirmed the findings. Ultrastructural tracer stuoies of HRP distribution did not show overt differences between the two groups of animals in the screening type of comparison carried out.

In addition to image analysis and protein leakage findings the ultrastructural studies were the first to show unidentified wall-free organisms in the bronchi of guinea pigs; there was a remarkable attachment to microvilli and an association with the basal portion of cilia. An extensive attempt to isolate and culture these organisms by special L-phase and mycoplasma media was unsuccessful, despite the cooperation of an expert pre-eminent in these areas of culture work.

The diphosphoglycerate content of red blood cells was measured in a colony of guinea pigs. Those exposed to continuous 0.36 ppm NO_2 for 1 week were found to have a significant increase ($p < .05$).

Support in part was given to a few projects tangential to the research direction of this program. The projects were opportunities to obtain new kinds of measurements of lung cell vigor and viability, using material supplied by an independent tissue culture program.

The overall findings from the image analysis and protein quantitation studies imply that both structural and functional alterations of the lung can be produced by supraambient and ambient levels of NO_2 exposure of guinea pigs and mice. An increase in numbers and size of Type 2 pneumocytes is believed to represent a corresponding loss of Type 1 lung cells, and a leakage of protein is in accord with the concept that the Type 1 cell is a critical barrier for fluid and protein leakage in the alveolar spaces. Changes in diphosphoglycerate content of red blood cells and increased content of protein in the urine indicate a systemic effect of NO_2. Other aspects of the work point to a role for NO_2 in placing additional burdens on the host defense system, in particular possibly providing an opportunistic circumstance for the pathologic emergence of organisms indigenous to the lung.

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ABBREVIATIONS

Å -- Ångstrom
BSS -- balanced (or basal) salt solution
C° -- Centrigrade
cm -- centimeter
cpm -- counts per minute
DPG -- 2,3-diphosphoglycerate
DAB -- diaminobenzidine
EM -- electron microscopy
Enzymes:
  AcPase -- acid phosphatase
  AtPase -- adenosine triphosphatase
  G6PD -- glucose-6-phosphate dehydrogenase
  HRP -- horseradish peroxidase
  LDH -- lactate dehydrogenase
  MDH -- malate dehydrogenase
g -- gravity units
GTA -- glutaraldehyde
^3H -- tritium
hrs -- hours
^3H-TdR -- tritiated thymidine
K -- thousand
M -- molar
ma -- milliampere
ml -- milliliter
mV -- millivolt
μ -- micron
μCi -- microcurie (10^{-6} Ci)
μmol -- micromole (10^{-6} Moles)
nM -- nanometer (10^{-9} meter)
NO_2 -- nitrogen dioxide)
PAGE -- polyacrylamide gel electrophoresis
PMI -- postmortem interval (delayed explantation in vitro)
ppm -- parts per million
RBC -- red blood cells
RH -- relative humidity
rpm -- revolutions per minute
Statistical data:
  X_1 -- number of Type 2 pneumocytes per microscopic field
  X_2 -- number of alveoli in the same field
  Y -- X_1/X_2
  T -- treatment
  D -- duration
  L -- lung lobe
  TDL -- treatment-duration-lobe
  S -- lung section
  ATDL -- animal-treatment-duration-lobe
  X_2^* -- area of each slide occupied by the alveolar wall
  Y^* -- X_1/X_2^* ratio of cells to baseline
T_3-T_6 -- from 3 to 6 congregating macrophages per target cell
UV -- ultraviolet light

SECTION 1

INTRODUCTION

The lung and other organs of the body can sustain an enormous amount of damage, and especially loss of tissue, without an overt effect. For example, an entire kidney can be donated for transplantation purposes without any clinical sign or patient awareness of impaired kidney function. Similarly, it appears that over one half of the lung must be lost before the loss comes to clinical or patient attention, and even before pulmonary function tests first become positive. The reason such losses are inapparent is the fact that the body in general has great compensatory mechanisms and an extraordinary amount of cellular and tissue reserves. Thus, signs of clinical disease (morbidity), or death itself (mortality), will generally be very crude discriminants for use in establishing air quality standards. While attention to exacerbation of illness can provide more sensitive and useful data, an evaluation of air quality demands that attention also be given to subclinical cellular alterations that deplete the functional and structural reserves of organs which are the primary targets for air pollutants. We have termed an abnormal loss of reserves "hypeinopenia." When this covert loss of reserve reaches a point where a person becomes particularly susceptible or vulnerable to bodily insults and illness in general, that person can be said to suffer subclinically from "morbility."

There can be no question that everyone, regardless of his or her environment, is to some extent losing cellular and tissue reserves of all organs. The important question is what is the baseline in the well population in terms of alveolar cells and alveoli that have been lost, and what correlation can be made with the various ambient atmospheres across the country.

The projects we have proposed over the past several years have been specific attempts to provide data on hypeinopenia as found in two animal models, the guinea pig and the mouse, and with respect to one of the air pollutants we considered to have high potential for an adverse health effect, namely nitrogen dioxide (NO_2). The principal objectives were to obtain quantitative measurements that would reflect physiopathologic alterations that could be related to the problem of air quality for human populations. Towards this end, we applied our experience with examination of human lung biopsies to a search for discriminants in an animal model that would be highly relevant for the human experience. We noted, as have others, that a loss of Type 1 cells and a replacement by Type 2 pneumocytes (and/or Clara cells) is a common denominator and early finding for a great variety of human lung disease. We considered edema and protein loss to be additional early, common denominators. Thus, the proposed projects have been based on animal models that permit exploration of these specific alterations, i.e. a population shift in the alveoli of the lungs and increased capillary permeability following air pollutant exposure. Nitrogen dioxide (NO_2) was singled out as one of the most important of the air pollutants for study, both with respect to its high potential for adverse health effects and as a prototype pollutant for the development of new methodologies that can later be applied to other pollutants, singly and in combination.

The results of our studies have been submitted as annual progress reports. They include a number of new discriminants we developed for the measurement of early pathophysiologic alterations following supraambient and ambient levels of NO_2, applied continuously and intermittently over short term periods of up to three months. More specifically, the efforts include quantitative measurements of numbers and size of Type 2 pneumocytes by image analysis, measurements of protein leakage in lung lavage fluid by quantitative electrophoresis and scintillation counts of tritiated albumin, measurements of protein content of urine, ultrastructural studies, and other approaches as presented elsewhere in this report. The results can also be found in our publications which we cite; the reprint sets were submitted earlier with the the progress reports.

SECTION 2

CONCLUSIONS

The purpose of these studies has been to develop highly sensitive discriminants for evaluating the adverse health effects of air pollutants, beginning with an investigation of the single pollutant, nitrogen dioxide (NO_2). New methodologies were innovated as a means of detecting early structural and functional changes in the lungs of guinea pigs and mice exposed to supra-ambient and ambient levels of NO_2. The results from the use of these new methodologies confirm our working hypothesis that the quantitation of cell population shifts in the lung, in conjunction with quantitative measurements of alterations in capillary permeability, are highly sensitive tests for assessing adverse health effects.

The results of tests of cell population shifts with quantitative image analysis (where guinea pigs were exposed to 2 ppm NO_2 for 7 days, 14 days, and 21 days) demonstrated that highly sensitive and versatile measurements can be achieved. Specifically, the initial study showed a high coefficient of correlation ($r > 0.8$; $p < 10^{-6}$) with hand counts of the numbers of Type 2 pneumocytes expressed as a ratio to the numbers of alveoli in the same lung sections. As with the hand count, the lungs of the exposed animals showed a greater number of Type 2 pneumocytes per alveolus ($p < .05$) than the control animals. However, the relationship to duration of treatment shown by the hand count ($p < .025$) was found by automated measurements to be of borderline significance ($p < .1$), a finding subsequently brought to significance by new measurements with an improved methodology (see below).

In a subsequent study of the image analyzer, a number of improvements were made in the methodology to include an automated stepping stage, the use of phase contrast images of the walls of the lung for quantitation, and a fully automated determination of Type 2 pneumocyte/alveolar wall ratios. High correlation coefficients were found in two specific areas: 1) between hand and automated cell counts ($r > 0.7$; $p < 0.001$); and 2) between hand counts of alveoli and automated wall area ($r > 0.56$; $p < 0.002$).

Final refinement of the image analysis methodology formed the foundation for an experimental model use of this objective, quantitative approach. A detailed analysis of the data obtained showed high correlation coefficients for two independent analyses of hand count and automated data ($p < 10^{-6}$), and also demonstrated that there were two significant effects, $\underline{NO_2 \text{ treatment}}$ ($F = 6.93$, $p < .02$) and $\underline{\text{duration of } NO_2 \text{ exposure}}$ ($F = 5.33$, $p < .05$).

The automated image analysis was expanded to include Type 2 pneumocyte cell size as well as cell numbers. From this study, it was found that a significant size increase for the exposed animals occurred ($p < .025$) as a result of the 2 ppm NO_2 exposure, and that the duration of the exposure (7, 14, and 21 days) was also significant ($p < .05$). This study, and the image analysis studies that preceded it, show that a study of the micro-ecology of the lung is both a highly sensitive and meaningful means of detecting early adverse health effects, as is well recognized for studies of the ecology in general.

To achieve other means of detecting early adverse health effects, and for the purpose of achieving correlations with image analysis studies, a number of physiologic methods were developed. The first of these pathophysiologic methods was a disc gel (PAGE) measurement of the protein content of the lung lavage fluid of guinea pigs exposed continuously to 0.4 ppm NO_2 for a one week period. Higher levels of protein characterized the lavage fluids of the exposed group of animals ($p < .001$). This methodology represents a relatively simple means of detecting a very important pathophysiological alteration, one that is a common denominator at the very early stages of a great many lung diseases.

In a subsequent study, two experiments were carried out to compare the protein content of NO_2 exposed guinea pigs to that of control animals: a) continuous 0.5 ppm NO_2 for one and two weeks, and b) intermittent 0.4 NO_2 (4 hours per day) for 9 ± .1 day showed an increase content of protein by PAGE in the urine of the exposed animals ($p < .01$ and $p < .05$ respectively for the two experiments). This preliminary study particularly warrants follow-up investigations and confirmation since it implies a systemic effect by exposure to NO_2 atmosphere at an ambient level (0.4 ppm) of NO_2.

The study of increased capillary permeability and protein leakage was expanded through the development of a new methodology, namely the labeling of plasma protein as it entered the lung, i.e. the use of a fluorescent labeling compound for primary amines. The results of the study showed that continuous 0.47 ppm NO_2 for ± 2 days was responsible for an increase of labeled protein in the lungs of the exposed animals ($p < .025$). This approach was used to measure not only increased content of protein in alveolar fluid, as demonstrated in the earlier studies, but also increased content of protein in cells, tissues, and lymphatic channels of the lung. This method was innovated in response to a need for evaluating the bidirectional cell transport in the lung of protein (plasma and cell breakdown origin) in the lung.

To expand the fluorescamine studies and also to achieve ultrastructural correlations, the molecular probe, horseradish peroxidase (HRP), was used to evaluate independently the influence of continuous 0.5 ppm NO_2, intermittent 0.8 ppm NO_2, and intermittent 0.6 ppm NO_2. In the first of the two experiments, the HRP content of lung homogenates (following intravenous injection of HRP and a testing delay for blood clearance) was found to be elevated ($p < .05$ in both exposed groups of animals following three weeks of exposure. No differences were found after six weeks. In a third experiment, an exposed group of animals again showed a higher HRP content ($p < .025$) but only after six weeks of NO_2 exposure. In one of the experiments, a difference was noted ($p < .05$) for just one of the experimental sets, the exposed groups again having a higher HRP content than that of the controls. The studies require elucidation.

A number of other experiments were carried out during the investigation. They include the following: a) the first ultrastructural demonstration that an unidentified wall-free organism appeared to be a common inhabitant of the bronchial mucosa of the guinea pig. This organism, with structural variations from elementary bodies to budding forms, was characteristically attached to microvilli and often located in large numbers at the base of cilia. These organisms are believed to represent a special life cycle for a bacterium (L-phase), mycoplasma, or some other organism. Our working hypothesis is that NO_2 may provide an opportunistic circumstance for the organisms to develop walls, or in some other way to become pathogens; however, the preliminary work did not show a relationship between NO_2 exposure and these organisms. An intensive effort to isolate and culture these organisms with special L-phase and mycoplasma media was unsuccessful; b) the diphosphoglycerate content of red blood cells of guinea pigs exposed to 0.36 ppm NO_2 for one week was found to be higher than that of the control group ($p < .05$).[2] This again is a preliminary finding requiring confirmation, but suggesting that NO_2 may have adverse effects outside of the lung, and in this instance, on the binding properties of red blood cells for oxygen; c) the project contributed a relatively small part of support to other studies where credit for the support was acknowledged in the report. These include studies with macrophages in tissue culture and, in particular, the measurement of lung cell outgrowth in tissue culture by a quantitative methodology involving the uptake of tritiated fucose and leucine followed by liquid scintillation counting. The published reports showed statistically significant differences between the control and exposed groups of animals.

SECTION III

RECOMMENDATIONS

The results of the studies strongly support the working hypothesis that it is the depletion of cell reserves by air pollutants that is the key to the establishment of air quality standards. A critical need is data on the acceleration of the rate of cell reserve depletion caused by exposure to ambient levels of pollutants. Only a beginning has been made in this direction. Of great pertinence, the hallmark of the human emphysematous diseases is the covert loss of over 80% of lung tissue before the loss comes to clinical attention. We consider image analyzer quantitation an important advance in detecting very early loss of Type I lung cells, and believe that the foundation we have established(20,6) should be expanded into studies of the human well populations as well as into comprehensive animal model investigations. The very high sensitivity of image analysis quantitation that can be expected with large volume quantitation, and the demonstration of pathophysiologic alterations at levels of NO_2 as low as 0.4ppm, attest to the feasibility and realistic nature of the recommendation. Some of the immediate questions raised are: 1) what is the lowest level of NO_2, singly and in combination with other pollutants, that will cause detectable structural and functional alterations? ; 2) are the alterations that result reversible? ; 3) what are the health effects implications of the alterations? In the latter respect, does protein leakage and Type 2 cell hyperplasia indicate Type 1 cell damage and loss that represents an early stage of emphysema? Note that early emphysema is in effect an "excessive" loss of lung cell reserves, and that image analysis quantitation now makes possible this type of measurement in both animal model and human studies.

There are other aspects of the microecologic evaluation deserving special attention, notably in vitro stress testing of cells for vigor or reserve functional loss. The functional reserves of cell populations will vary according to both inherited and acquired factors, and clearly play important roles in creating especially susceptible cell populations. One promising approach we have developed for measuring individual cell population reserves is the postmortem interval stress test, i.e. the capacity of the cells to withstand delayed explantation in culture. This type of dynamic approach should be expanded to include biochemical and ultrastructural correlations.

Lastly, air pollution is but one factor in perturbations of the microecology. The well recognized "constellation of events" from all factors demands some close cooperation between investigators working in diverse areas. Special efforts are needed to encourage more integration of work and the exchange of ideas. Our work with a probable special life cycle of an opportunistic bacterium had the valuable assistance of experts in mycoplasma/ L-phase cultures. Our work with molecular tracers would have benefitted by parallel studies carried out by others using organs other other than the lung that we supply . Note that our urine protein measurements indicate that NO2 very likely has systemic effects, and the DPG studies as well. Human studies of protein excretion-pollution relationships would seem to be a very important priority., and should be expanded to include immunologic and other newer assays. Quantitative work on the bidirectional transport of protein in the lung also deserves special support since a great increase in workload no doubt precedes the occurrence of functional and structural abnormalities which are themselves of a very subtle nature.

SECTION 4

MATERIALS AND METHODS

ANIMALS

For the most part, the studies have used Swiss-Webster mice from various vendors (Simonson, Charles River, and Hill Top Laboratories). For studies using guinea pigs, purchases were made from Camm Laboratories, and the animals were Hartley strain. On arrival, the animals were maintained in quarantine by the Vivarium for 7-10 days and were then transferred to a laboratory room maintained exclusively for our exposure chamber use. The mice were housed in standard plastic cages with stainless steel grids, in a bedding of wood shavings, and with a maximum of five mice per cage. The guinea pigs were housed in galvanized iron guinea pig cage racks, with a maximum of five guinea pigs per cage. For most of the experiments, a standard chow for the respective animals was provided with food and water ad libitum. In some instances, the guinea pigs were fed a rabbit chow to provide a low Vitamin C content diet.

EXPOSURE CHAMBERS

The cages used for the study were two identical cube units, 27 cubic feet in volume. The cages are wood construction, and the interiors have a complete epoxy layer. Air flow was regulated to provide approximately one turnover every two minutes, with a slight negative pressure from the exhaust fan used to provide air flow. The air was filtered through two units in tandum, a particulate filter and either a charcoal or Purafil filter. Both air intake and air exhaust were common to both units; the only difference was a mixing chamber to the air intake of the exposure unit. (In the final year of the program, a completely stainless steel exposure chamber with laminar flow was built to replace the epoxy unit). The cages were spaced within the chamber to provide a correspondence between cage numbers of the control and exposed animal groups. The bedding (or pans) was changed generally from two to three times a week, depending upon the need.

The temperature in both chambers was maintained at $23^{\circ}C \pm 2^{\circ}C$ and at ambient humidity (generally 50-65% RH). The humidity indicators used in the chambers were Abbeon AB-1-67.

POLLUTANT

In the study, a single pollutant nitrogen dioxide was used at various concentrations and at intermittent as well as continuous periods of exposure. The nitrogen dioxide was obtained from Matheson Chemical Company and it was added to a volume of silicone fluid (medical grade 360, 500 centistokes, Dow Chemical Co.). The methodology for this NO_2 delivery system was developed earlier in our laboratory and the details can be found in the published report (19). In brief, silicone fluid, an inert substance, will absorb NO_2 intermolecularly in an unusually large quantity (the precise amount is now the subject of an ongoing investigation). Once the NO_2 gas phase has equilibrated in the separatory funnel, and the concentration has been found to be satisfactory through monitoring, the NO_2 laden silicone is allowed to drip into the mixing chamber where the NO_2 is released by Venturi forces of the mixing chamber. The completely clear silicone fluid is collected in a glass container at the bottom of the mixing chamber, and is discarded. This delivery system is highly advantageous, particularly since the height of the silicone and air flow can be readily controlled, and thus the level of NO_2 established very easily maintained. The exhaust fan is connected to a constant voltage unit and the air flow periodically measured by a flow meter. Monitoring of the NO_2 was achieved by Saltzman fritted bubbler analysis with routine standardization and bubble meter monitoring of the fritted bubbler airflow. Periodic comparison of the NO_2 levels was achieved by loan use of chemiluminescent NO_2 detectors, and grab sample spot checks.

Lactate dehydrogenase identification of Type 2 pneumocytes for quantitative measurements

The methodology was first reported in a guinea pig study (20). In brief, the lungs are removed from the animals immediately after death by a lethal intraperitoneal pentobarbital injection. With the lung placed on a cold surface, 6% gelatin is instilled via the trachea to achieve inflation of the lung corresponding to the thoracic volume, and with slow, gentle pressure on the hypodermic syringe used for injection. Loss of the sharp angulation of the parenchymal markings was the immediate endpoint for cessation of injection. The gelatin was allowed to solidfy at 4°C and then the lungs were placed in a deep freeze for cryostat sectioning (-20°C). Cryostat sectioning was done at 15 micra, with guinea pig lungs in the coronal plane and mouse lungs in the horizontal plane. The frozen sections on glass slides were lyophylized and processed for the lactate dehydrogenase (LDH) reaction. The details can be found in the earlier report (20). In brief, a tetrazolium salt is used as the electron acceptor and is reduced to an insoluble formazan in the presence of the LDH enzyme, with lactate as the substrate. The formazan reaction product is most heavily deposited in the cytoplasma of Type 2 pneumocytes with a variable and entirely different type of deposition in macrophages and with negligible deposition in Type 1 and endothelial cells under the conditions of the reaction used.

Lung lavage

Immediately after death of the animal by intraperitoneal pentobarbital injection, the thoracic and abdominal cavities were opened and a portion of the anterior rib cage resected to provide a wide exposure of the lungs. The trachea was then prepared for cannulation by removing fatty tissue and free blood. A short horizontal slit was made in the trachea approximately 5 mm below the larynx to permit the insertion of a 14 gauge 4 inch long cannula (for the guinea pig) with attached 20 ml syringe, the cannula and syringe having been previously filled with Gey's balanced salt solution. For the mice, a 1 ml syringe was used with an attached 22 gauge cannula. The cannula was securely tied in place by two encompassing sutures. The lungs were gently lavaged over four complete cycles of injection and retrieval, with the lungs being inflated to a point where they occupied nearly completely the thoracic space. For the guinea pigs, 10 ml of BSS were introduced and 7 ml recovered. For the mice, 1 ml of BSS was introduced and 0.7 ml recovered. At the completion of lavage, the cannula was removed from the syringe and the contents of the syringe entered into a chemically clean screw top vial. The specimens were immediately placed in a refrigerated centrifuge (4°C) and the supernate stored at 4°C before subsequent testing (4).

Gel electrophoresis

For the gel electrophoresis procedure, a 125 Lambda sample of each lavage fluid was introduced into the loading gel portion of the preparation. The method of Ornstein (21) was followed with the exception that sucrose was omitted from the loading gel, the tubes were rinsed in a detergent solution, and the order of preparing the vaious gels was reversed, i.e. separatory gel first, followed by stacking and loading gels. A bromophenyl blue dye was used in the buffer of the electrophoresis apparatus (BioRad) and a current of 4 ma was applied for 45 minutes or until the tracking dye extended 1.5 inches beyond the stacking-separating gel interface. The proteins were stained in 1% amino black for a minimum of one hour. The gel columns were destained for four hours and washed in a solution of 7% acetic acid with a current of 10 ma per tube. A gel scanner recording was made of each tube at a wave length of 559 mm, at 0.26 mm slit width, and with constant scan and chart recording speeds. Each gel electrophoresis run involved 12 specimens representing two matched animal pairs with triplicate samples from each animal. Additional runs were employed as needed. The scan recordings were quantitated by either of two methods: a) initially, the use of heavy weight, semitransparent drafting paper for tracing the scans, followed by weighing of the cut-out scans; b) planimetry; c) the use of an integrated chart recording. The gel measurements included those for LDH, ferritin, horseradish peroxidase (HRP), protein (Amido black and Coomassie blue), and other substrates (16,18).

Protein determinations

Protein analysis on lavage fluid, lung homogenates, and urine were carried out using a Lowry procedure (22,23) and polyacrylamide gel electrophoresis (PAGE).

Electron microscopy

Our ultrastructural methodology has been covered in earlier reports (1 ,24). In brief, portions of bronchus and lung were cut into 1 mm cubes, fixed in cacodylate buffer 2% glutaraldehyde, postfixed in 1% osmium, dehydrated in ethyl alcohol, embedded in araldite , epon, or other resins (araldite-opon; Vestopal W), double stained after thin sectioning (500 Å) with uranyl acetate and lead citrate. The sections were examined with a Philips 301 electron microscope, or an Hitachi HU7.

Horseradish peroxidase permeability studies

Following each exposure period, the mice were heated for 30 minutes under an infrared lamp (37°C) in order to dilate the tail veins. They were then given an intravenous injection of low to high dose HRP, depending on the experiment. The high dose was 0.5 mg of HRP (Sigma type II, 50 mg/ml of BSS) per gram body weight, using a 1 ml syringe and a 27 gauge needle. The low and other doses are covered in the Results. Each animal was killed at specified postinjection periods for the permeability studies. Immediately after death, blood was collected from the jugular veins and spun down in a microfuge for five minutes. The serum was placed in a deepfreeze at -85°C. The right ventricle of the heart was perfused with 20 ml BSS (22°C) until maximal blanching of the lung was achieved, generally a perfusion period of one minute. The lungs were removed en bloc, placed on a BSS moistened gauze sponge, compressed gently two to three times by fingertip pressure to express fluid from the vascular bed and air spaces, placed in a tared vial, weighed and then frozen in a deepfreeze at -85°C. A similar perfusion and handling procedure was carried out for the right kidney.

For the HRP assay, frozen lung tissue was homogenized by means of a Polytron in 2 ml of BSS (5°C) containing 10% isopropanol and 10^{-4} phenomethylsulfonylfluoride. The homogenate was spun down in duplicate 25 micro liters and 50μL samples of supernate were loaded on gels for PAGE analysis. A portion of the supernate was also used to determine protein content by Lowry's method, and also for comparative studies with the enzyme kinetic assay for HRP (see below). PAGE was run at 2-3 mA per gel for three hours.

The gels were incubated in a benzidine-guaiacol stain for 30 minutes in the dark at 25°C, and then were fixed in 7% acetic acid. Immediate scanning was done on a Beckman Acta 3 spectrophotometer at 525 nM with a scanning speed of 1.5 cm/min. The tracings were measured through the use of an integrated recorder with settings at 10 mV at 12,000 cpm, with a chart speed of 4 cms/min. and a span of 2. Measurements were obtained of HRP content of lung, kidney, and serum (16,18).

Enzyme kinetic assay for HRP

Assays were carried out for both lung homogenates and for portions of gels containing the HRP band (as indicated by a corresponding stained gel sample). Also included in the enzyme kinetic assay was one of the triplicate samples of serum and kidney homogenate, two processed for PAGE and one for the assay. For the gels, 1.5 cm portion of the gel containing the HRP band was homogenized in 1 ml distilled water using a Polytron, and the homogenates spun at 1,000 rpm for 10 minutes in a refrigerated centrifuge. The samples were allowed to warm to room temperature and were assayed by a modification of the O-diansidine method. A supernate sample was placed in a 1.4 ml glass cuvette (path length of 1 cm) and 0.9 ml of substrate added to the 0.1 ml of sample. Following immediate mixing, the rate of color development at 460 nM was recorded using a Gilford spectrophotometer with a recorded chart speed of 2 inches per minute, a span of 1, a ratio of 1.0. A temperature of 25°C was maintained by a Lauder Model K2-R circulating water bath (25).

For the electronmicroscopic studies of HRP, several procedures were used. These included: 1) perfusion of the trachea with 2% glutaraldehyde, in an amount sufficient to expand the lungs to fill the thoracic cavity, followed by tying of the trachea and suspension of the lung in a 2% GTA solution for two hours. The lungs were then thinly sliced and the sections processed according to the DBA reaction, with control sections for endogenous peroxidase; 2) perfusion of the left ventricle with 3 ml of BSS followed by 3 ml of 2% GTA, and after five minutes of perfusion fixation in GTA, perfusion with 5 ml of DAB solution with suspension in DAB for 30 minutes.

Diphosphoglycerate determinations

The method for a simplified assay of 2,3-DPG was developed earlier (26). In brief, 10 ml of guinea pig heart blood, with potassium oxylate as an anticoagulant, was centrifuged at 4°C and the packed RBCs resuspended and washed in isotonic saline. The RBCs were hemolyzed with cold distilled water and the protein component precipitated with trichloroacetic acid. The filtrate was absorbed with charcoal to remove all but two forms of phosphorus, inorganic phosphorus and that bound to 2,3-DPG. The filtrate was then tested independently for the latter two compounds by the means of a colorometric assay based on a molybdenum compound. Duplicate samples of 0.25 ml and 0.5 ml aliquots were tested (5).

Acid phosphatase

Lung tissue was rapidly sliced into 1 mm cubes in a drop of 0.4 M sucrose on a waxed plate and then placed into an homogenizer tube containing 1.4 ml of cold 0.4 M sucrose for each 100 mg of lung tissue. After 10 complete passes of a glass pestle at 600 rpm, the homogenate was centrifuged for 10 minutes at 140 g and the supernate obtained diluted to its original volume with cold 0.4 M sucrose at pH 7.2. Two aliquotes of the supernate were overlayed onto fresh gradient tubes, and each contained 26 ml of a linear gradient ranging from 0.149 to 2.255 M sucrose (5% to 60% sucrose), and this in turn was overlayed with 0.1 M sucrose to within 1/8 inch of the top of the gradient 2. Using a SW 27 rotor, the preparation was centrifuged for 150 minutes at 22K rpm (70Kg) in a Beckman L-350 ultracentrifuge prechilled to 1°C. One of the two gradients was fractionated using a peristaltic pump and fractionater. The fractioning pipe was inserted from the top of the gradient tube and lowered by 64.5 turns of the control handle. This placed the tip of the pipe approximately 1 nM below the lowest observed band. At slow pump speed, 20 drops (1 ml) were collected for each fraction and 20 fractions were collected from each gradient tube. Acid phosphatase (27) and protein (28). Assays were done on all 20 fractions from each animal specimen. For the enzyme assay, 0.2 ml of each fraction was added to 0.5 ml of substrate, and the mixture was incubated at 37°C for 30 minutes, following the addition of 5 ml of 0.1 NaOH, a readout at 405 nM was obtained (7,17).

Ferritin studies

Ferritin tracing was used for both PAGE studies and electron microscopy. All animals were given an intravenous injection of horse spleen ferritin (Worthington) via femoral or tail veins (1 Gm/Kg and.5mg/Gm body weight of guinea pigs and mice respectively). The methodology followed was essentially that of Farquhar et al (28). Both the EM and PAGE procedures were essentially identical to those described above (29).

Other procedures

A number of other procedures were carried out during the period of support. Since the data have not been published only brief mention is made of the methodology. The main reason for incompletion of the studies was interruption of the work when the postdoctoral fellows (Drs. Yuen and Hanson) carrying out the investigations could not be assured of continuing support and accepted positions elsewhere. Dr. Charles Hanson took on the assignment of specifying the species of proteins being found in lavage fluid and urine as as well as other biochemical aspects. His work involved PAGE, column separation of proteins, lung membrane preparations, and SDS-PAGE analyses. Other incompleted methodologies include fluorometric assays of fluorescamine (carried out by Lester Layfield), FITC labeled plasma proteins in lung homogenates, and ferritin tracing studies for kidneys and lungs (Dr. Yuen).

Statistical analyses

The analyses generally use a Student's t test for independent variables and a two-factor analysis of variance. The details of the analyses are provided in the published reports. The statistical approach was under the direction of Stanley P. Azen (a co-author of the book "Statistical Analysis, a Computer Oriented Approach," Academic Press, New York, 1972) and the problems of developing and correlating the various methodological quantitative analyses have been covered in our collaborative report (12).

Image analysis

With the cooperation of Mr. Brian Partridge of the Cambridge Instrument Company (Imanco) and Mr. Gus Faulhaber of Leitz, the Quantimet 720 and the Leitz Clasimet respectively, were used for a part of our work. The details can be found in the published report. In brief, a very practical and highly reproducible means of obtaining large volume quantitative measurements of Type 2 cells, alveolar walls, internal surface area, and other parameters have been demonstrated with the use of these instruments.

SECTION 5

RESULTS AND DISCUSSION

Three studies of continuous exposures to 10 ppm NO_2, 2 ppm NO_2, and combined 3 ppm NO_2 and 5 ppm SO_2.

Wall free organisms on bronchial mucosa of guinea pigs; Ultrastructural study

The three studies, all with guinea pigs, were as follows: three pairs control and exposed (continuous 10 ppm NO_2 for three weeks); six pairs control and exposed (continuous 2 ppm NO_2 for three weeks); and eleven control and exposed (3 ppm NO_2 and 5 ppm SO_2 continuously for three weeks). All the animals were studied for the presence or absence of the wall-free organisms discovered on an earlier screening examination of the electron microscopic sections. The midportion of the bronchus of the right middle lobe from each animal was excised under aseptic conditions and portions were taken in a specific sequential order for the following studies: electron microscopy, bacteriologic culture with trypticase soy broth, and cultures for L-phase organisms and mycoplasma using special media applied with and without thallium acetate by Dr. Leonard Hayflick of Stanford University.

Of approximately 500 organisms found in the bronchi of ten guinea pigs, 347 were wall-free organisms (Fig. 1) and the others mature bacteria. With respect to the occurrence (Fig. 1) of the organisms in the animals, no differences were found between control and exposed animals, i.e. six of 19 control and four of 19 exposed (Table 1). Further, five of ten animals having wall-free organisms in their bronchi also showed mature bacteria. Of 30 animals where wall-free organisms were not found in the bronchi, five did show intrabronchial mature bacteria. The wall-free organisms were located primarily at the base of cilia and in between microvilli. Particularly noteworthy was the apparent attachment in some instances of the wall-free organisms to both cilia and microvilli. Elementary bodies were also associated with wall-free organisms. The specific ultrastructural details have been reported (1). In brief, the wall-free organisms ranged in size from 281 nm to 950 nm, with most of the organisms (92%) less than 500 nm in diameter. The plasma membranes (trilaminar membrane) had the following measurements, from external to internal surfaces, 27 Å, 20 Å, and 27 Å. A floccular, irregular thickening of the outer surface generally measured less than 150 Å in thickness, the so-called "fuzz" layer of wall-free organisms.

Bacterial, L-phase, and mycoplasma culture studies

Of 160 L-phase cultures of lungs, 27 of tracheas, and 48 of bronchi, no conclusive L-phase organism was identified. In a few instances, cultures suggestive of L-phase organisms were studied by electron microscopy but a heavy growth of bacteria around the suspected colonies made selection difficult and obviated any attempt at confirmation.

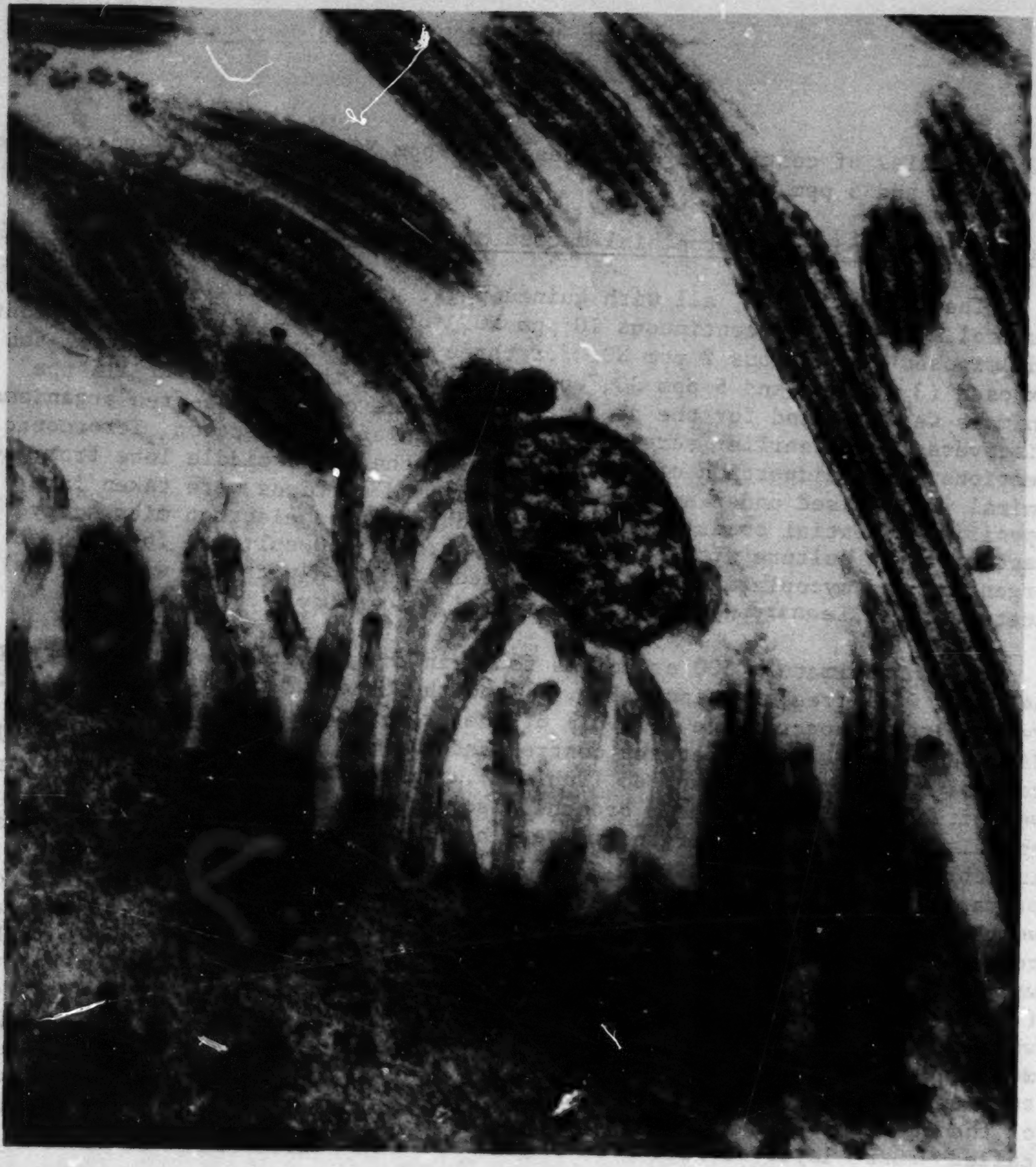

Figure 1a. Bronchus of a guinea pig exposed to a combination of 5 ppm SO_2 in combination with 3 ppm NO_2. Several microvilli appear to be attached to this wall-free organism, with one microvillus exhibiting an intimate juxtaposition to 25% of the circumference of the organism. The two, small, spherical, structures at one pole of the organism are believed to represent elementary bodies. The cytoplasm of the two cells underlying the organism had focal areas of presumable degeneration, with loosening of the cytoplasmic matrix as shown here. The bar is 0.5 μ in length; the organism, one of the largest found, is 500 nm. x 775 nm. in cross diameters in diameter.

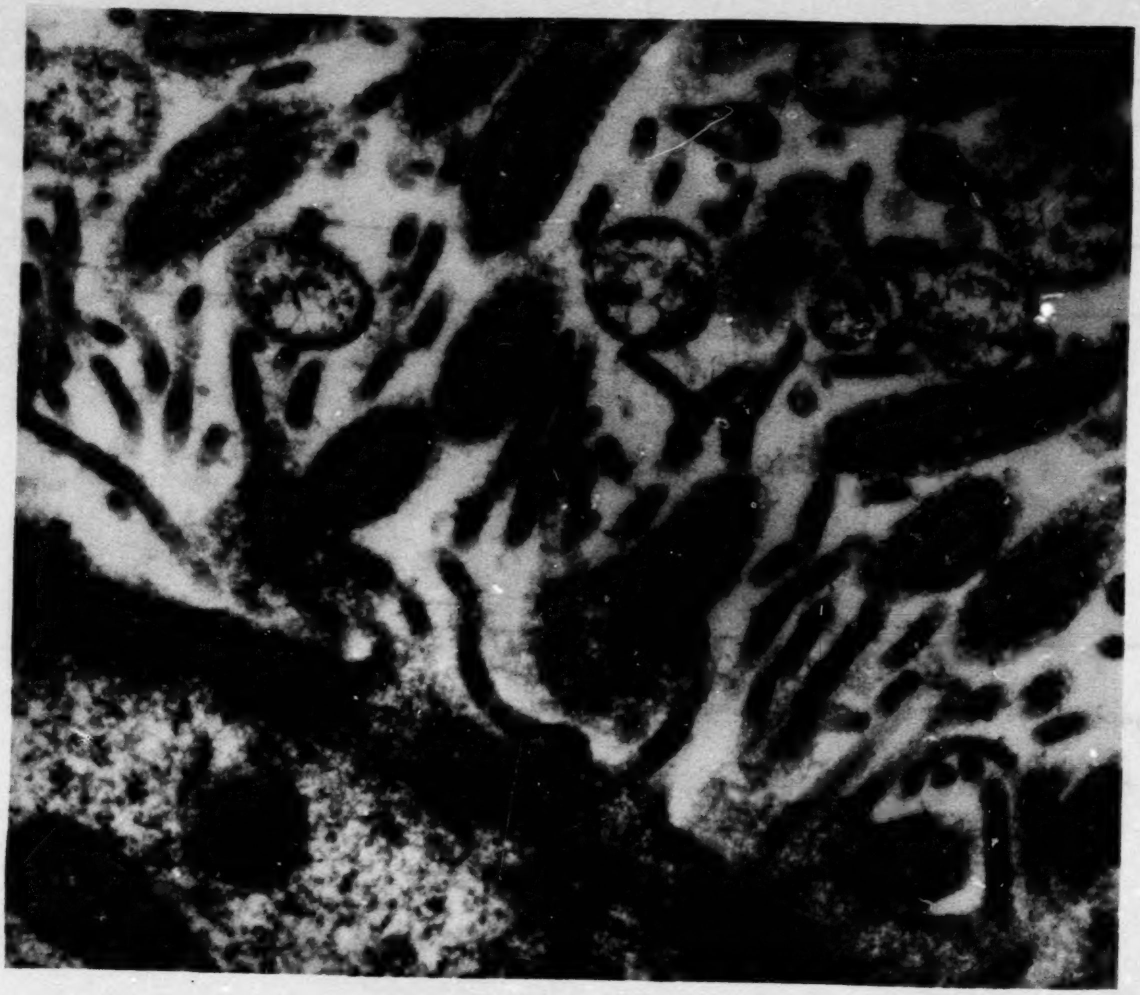

Figure 1b. Bronchus of a guinea pig exposed to 2 ppm NO_2. Two of the organisms show bud-like processes, with one process containing a fairly large dense body (arrow). The cytoplasm of the underlying cell shows a diffuse looseness of the cytoplasmic matrix. There is also a suggestive degeneration of mitochondria. The bar is 0.5 μ in length.

Table 1
Number of Animals in which Wall-free Organisms were Observed*

Experiment	Control	Exposed
1	0/3	1/3
2	1/6	2/6
3	5/10	1/10
Total	6/19	4 of 19

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* Numerator represents number of animals in which wall-free organisms were observed; denominator represents number of animals in study.

Summary

This study resulted in the first published report on the spontaneous occurrence of wall-free organisms in the lungs of guinea pigs and their association with microvilli and cilia (1). The demonstration of an intimate relationship between the wall-free organisms and microvilli suggests both a means of extraordinary resistance to bronchial clearance and a special event in the life cycle of this so far unidentified organism. A relationship of exposure of the animals to air pollutants was not established; the numbers of animals included in the study was insufficient for a definitive evaluation of air pollutant influence, i.e. increased incidence or conversion.

Continuous 2 ppm NO_2 exposure for 7, 14, and 21 days
Hypertrophy of alveolar wall cells in guinea pigs

Semi-automated quantitation of area of Type 2 pneumocytes and alveolar wall cell area

Twenty-four male guinea pigs were equally divided into control and NO_2 exposed animals and the right lower lobes of four pairs each were quantitated at each of three exposure periods. The automated stepping stage of the microscope of the image analyzer provided seven microscopic fields (0.38 x 0.47 nm) for Type 2 and alveolar wall measurements. The measurements included: 1) the sum of areas of the lactate dehydrogenase(LDH) positive alveolar wall cells; 2) number of alveolar wall cells; 3-6) numbers of wall cells remaining after step-wise increases in the sizing threshold, i.e. the number of cells remaining when the analyzer sequentially excluded cells with linear dimensions of less than 5 micra, 10 micra, 15 micra, and 20 micra respectively. The data were printed out by a desk calculator interfaced to the image analyzer (2).

The data were based on ratios of the sums of alveolar wall cell areas divided by the sum of the number of cells counted in that field. This was done for each of the seven fields. The data have been summarized in Tables 2 and 3. In brief, an analysis of variance showed significant differences between the NO_2 and control groups of animals on the basis of the NO_2 exposure ($p < .025$) and also for the duration of the experiment, i.e. greater change after longer exposure ($p < .05$). The specific values for each of the animals has been presented in Figure 2.

Summary

In a study of the lungs of 24 guinea pigs, half of which were exposed to 2 ppm NO_2 by image analyzer quantitation of Type 2 pneumocytes, an increase in the size (hypertrophy) of the Type 2 pneumocyte occurred in the NO_2 exposed animals as opposed to the controls ($p < .025$). The duration of exposure to the NO_2 also appeared to influence the size of these cells ($p < .05$). The results were based on an image analyzer quantitation of 9,824 lactate dehydrogenase positive alveolar wall cells.

The precise meaning of the hypertrophy is not clear at this time. However, it is well recognized that both an increase in the numbers and size of the Type 2 pneumocyte follows a great variety of insults to the lung tissues, and is considered one of the early signs of damage to the Type 1 pneumocyte. The findings implicate some impairment of gas exchange secondary to both the

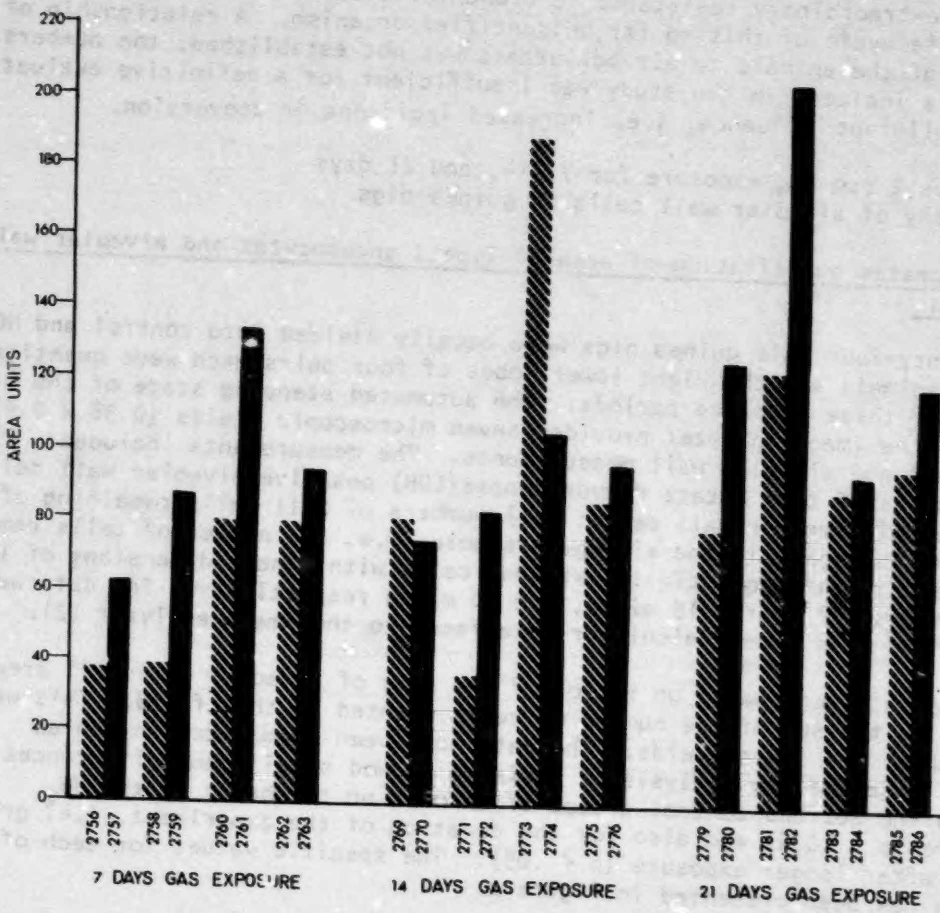

Figure 2. Average area-cell ratio, in arbitrary units, for each of the 24 animals; 2773 and 2782 appear as outliers, but high value of latter may be due to experimental treatment. Treatment and duration both significant at $p < .05$ level. Treatment was significant for seven-day group and, with animal 2782 included, for 21-day group also (solid bars, exposed animals; striped bars, control animals).

Table 2 – Absolute Data*

			Area (sq μ)	Diameter (μ)
Cell Counts	Control	4,986		
	Exposed	4,838		
	Total	9,824		
Mean Areas & Diameters, With Standard Deviations	Control			
		¢ 2,773	56.50 ± 27.9	8.27 ± 2.0
		$ 2,773	50.18 ± 18.2	7.86 ± 1.5
	Exposed			
		¢ 2,782	71.31 ± 24.7	9.42 ± 1.5
		$ 2,782	65.41 ± 14.6	9.08 ± 1.0

*Number of cells counted in 12 control and 12 exposed slides; absolute values (in microns of the marginal means of the average area-cell ratio and diameter-cell ratio for control and exposed animals. Relative areas are given in Table 2 and displayed in the Figure; conversion from area to diameter assumed circularity of cells. As possible outliers, animals 2773 and 2782 were both included and excluded in the calculations.

Table 3 – Means and Standard Deviations of the Average Area-LDH-Positive Cell*

		Duration of Experiment (Days)		
		7	14	21
Treatment	C	58.8 ± 24.2 n = 4	68.4 ± 26.9 n = 3	97.3 ± 19.0 n = 4
	X	94.2 ± 29.9 n = 4	90.9 ± 14.1 n = 4	113.3 ± 16.7 n = 3†

*Relative values in arbitrary units of the means of the average area-cell ratio for the six different treatment-duration groups. The two outliers were omitted; the dagger indicates the substituted value when the outlier in the 21-day exposed group is included in the analysis.

†When n = 4, this value is 136.1 ± 47.7.

presumed alteration of Type 1 lung cells and the barrier effect to gas exchange of the thickened Type 2 pneumocyte.

Exposure of guinea pigs to 2 ppm NO_2 for 1, 2, and 3 weeks

Image analyzer quantitation of numbers of Type 2 pneumocytes

In an earlier study (30), randomly selected fields of 141 lung sections from 24 guinea pigs (half of which had been exposed to 2 ppm NO_2) were examined and a ratio determined of the numbers of lactate dehydrogenase positive Type 2 pneumocytes to the numbers of alveoli. The methodology involved projecting 141 35 mm slides on a paper screen and having a technician count by hand, with pencilled marks for checking off structures counted, all cells and alveoli found in the 25X microscopic field photographed. In the present study, the same photographic slides were quantitated by image analysis, using a Quantimet 720 image analyzer for the detection of LDH positive pneumocytes and the alveolar counts as previously determined by hand counts. The findings are presented in Tables 4 to 6, and Figure 3. The correlation analysis (Fig. 4) demonstrated a highly significant linear relationship between the average hand count and each of the two automated cell counts that were carried out ($p < 10^{-6}$). The correlation coefficient between the two automated cell counts was 0.945 with a 95% confidence interval of 0.92 to 0.97. The calculated regression equations for each set of data are shown in Table 5.

Summary

A prior hand count of 56,587 Type 2 pneumocytes and 27,720 alveoli was repeated with the use of an automated image analyzer. Type 2 pneumocytes (LDH positive) were again shown to be increased in a study of 141 photographic slides of 25X microscopic fields for lungs of 24 control and exposed (2 ppm NO_2) guinea pigs. A high coefficient of correlation was obtained ($R > 0.8$; $p < 10^{-6}$), and the time required for counting was reduced by a great order of magnitude. Further details can be found in the published report (3).

Exposure of guinea pigs to continuous 0.4 ppm NO_2 for one week.

Polyacrylamide gel electrophoresis (PAGE) for quantitation of protein content of lung lavage fluid

The study involved lung lavage of 18 white male, short hair Hartley strain guinea pigs, equally divided into control and experimental (0.4 ppm NO_2) groups. The results have been presented in a published report (4). In brief, a nested 2-factor analysis of variance (Table7a) showed a high level of statistical significance ($p < .001$ for the greater amount of protein found in the lavage fluid of the NO_2 exposed animals (Table 7b). Of further interest, the densitometric recordings of the lavage fluid PAGE findings showed a prominent peak (albumin) followed by two minor peaks believed to be a mixture of α and β globulins and λ globulins respectively (Fig. 5). The increases found were believed to be the result of a general rise in protein content rather than a selected increase in lower molecular weight proteins.

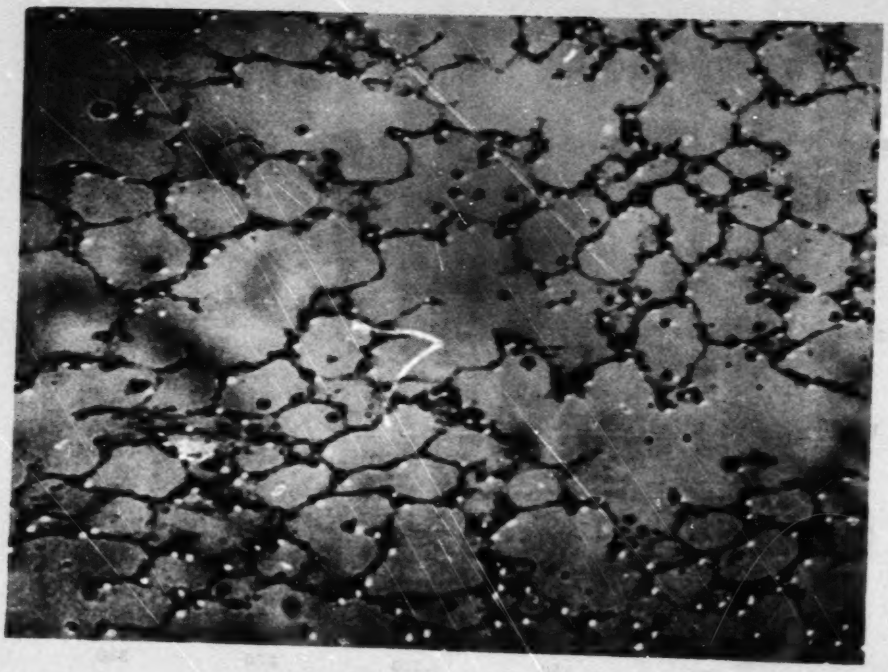

Figure 3. Phase contrast photomicrograph of linear alveolar structures, as detected by the image analyzer.

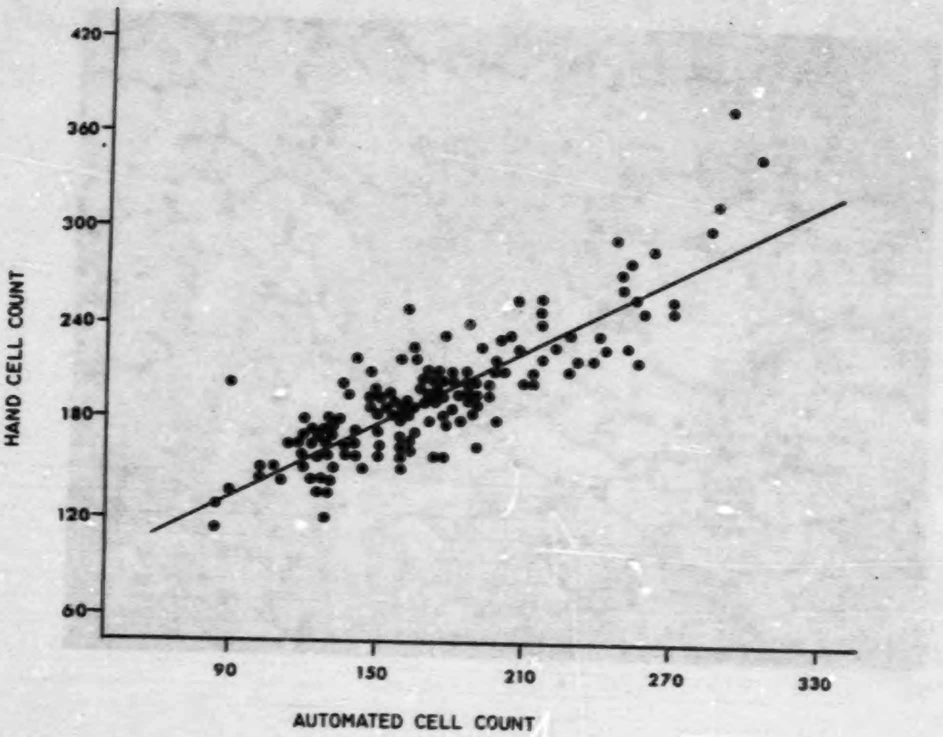

Figure 4. Plot of average hand cell count versus automated cell count No. 1.

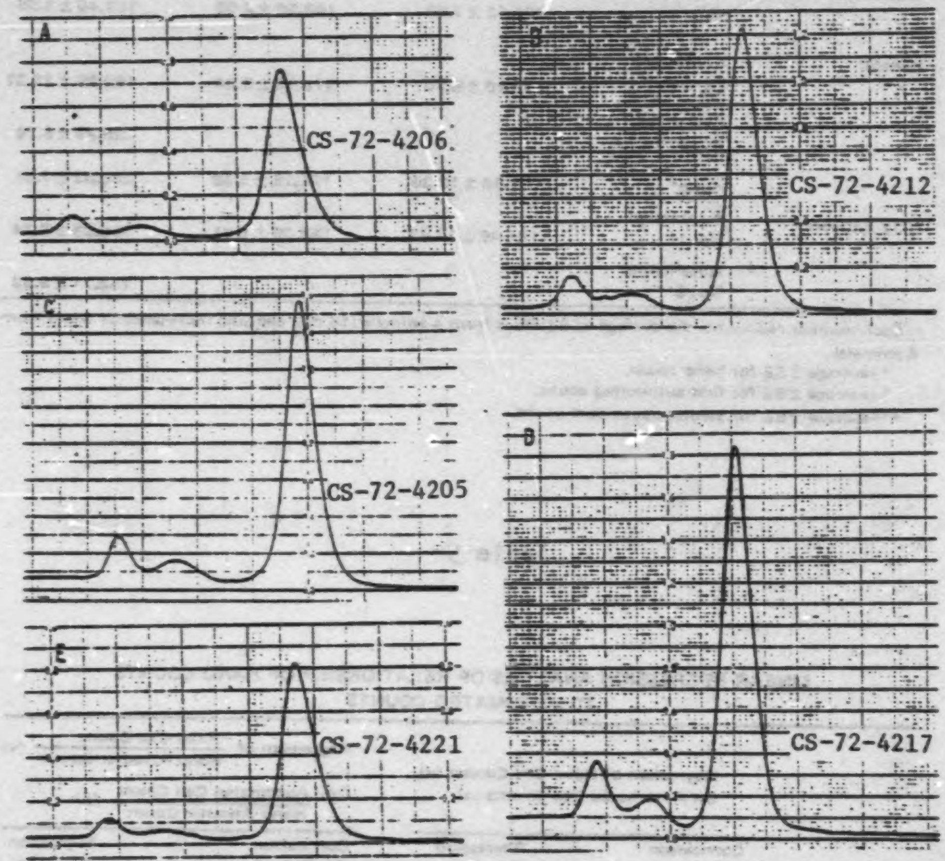

Figure 5. Densitometric recordings of lavage fluid disc-gel electrophoresis. A and B, Typical findings for control animal specimens. There is one prominent peak and two small following peaks, which are believed to represent albumins, a mixture of α- and β-globulins and γ-globulins, respectively. C and D, All of these peaks are accentuated, and the globulin peak is much better defined. E depicts exposed animal specimen which was an exception to consistently high values found. Total protein levels can be found in Table 1, i.e., animals C_2, C_3, X_1, X_7, and X_9, respectively. The levels are expressed in arbitrary units based on the weights of paper cutouts of tracings. Dominance of exposed animal values, with the exception of animal X_9 is evident.

Table 4

COMPARISON OF AVERAGES ± SE OF HAND COUNTS AND AUTOMATED COUNTS

Animal Group	Type of Count	Duration of Experiment *(days)*		
		7	14	21
	Hand*	209.92 ± 7.57	198.56 ± 8.00	187.40 ± 8.06
Control	Automated No. 1†	178.13 ± 8.70	178.13 ± 9.84	160.88 ± 10.21
	Automated No. 2**			128.79 ± 8.76
	Hand*	205.94 ± 12.38	178.28 ± 5.56	200.33 ± 7.51
NO_2 Exposed	Automated No. 1†	184.48 ± 12.48	158.29 ± 5.61	180.29 ± 8.74
	Automated No. 2**			158.71 ± 9.63

Each number represents the average of 24 slides from 4 animals (14 day exposed represents 21 slides from 4 animals).

*=average ± SE for hand count.

†=average ± SE for first automated count.

**=average ± SE for *second* automated count.

Table 5

LINEAR REGRESSION ANALYSIS OF RELATIONSHIP OF HAND COUNTS TO AUTOMATED COUNTS

	Regression of Hand Cell Counts (y) On Automated Cell Counts (x)		Regression of $\frac{\text{Hand Cell Count}}{\text{Hand Alveolar Count}}$ (y) On $\frac{\text{Automated Cell Count}}{\text{Hand Alveolar Count}}$ (x)	
	Correlation Coefficient	Regression Equation	Correlation Coefficient	Regression Equation
Automated Count No. 1 (n = 141)	0.853* (0.80, 0.89)	$y = 63.8 + 0.767x$	0.891* (0.86, 0.92)	$y = 0.807 + 0.803x$
Automated Count No. 2 (n = 60)	0.807* (0.69, 0.88)	$y = 94.6 + 0.683x$	0.887* (0.82, 0.93)	$y = 0.960 + 0.743x$

*$P < 10^{-6}$; numbers in parentheses are 95 per cent confidence intervals.

Table 6

ANALYSIS OF VARIANCE OF RATIOS DERIVED FROM AUTOMATED AND HAND COUNTS

Source	Degrees of Freedom	Type of Count	Mean Squares	F Ratio	P Value
Treatment (T)	1	Auto*	2.88	4.20	<0.05
		Hand	2.04	3.99	≈ 0.05
Duration (D)	2	Auto	1.83	2.67	<0.10
		Hand	2.28	4.46	<0.025
Lung Site (L)	1	Auto	0.006	0.007	NS
		Hand	0.191	0.372	NS
T x D	2	Auto	1.86	2.71	<0.10
		Hand	1.87	3.66	<0.05
Animal A (TDL)	41	Auto	0.686	9.30	<0.001
		Hand	0.511	7.98	<0.001
Section S (ATDL)	96	Auto	0.061		
		Hand	0.064		

*Automated count.

TABLE 7a. ANALYSIS OF VARIANCE OF PROTEIN CONTENT

Source	Degrees of Freedom	Mean Square	F Ratio	P Valve
Treatment (T)	1	.02630	16.34	< .001
Animal A (T)	16	.00161	41.82	< .001
Replicate R (AT)	34	.0000388	—	—

TABLE 7b. PROTEIN CONTENT OF LUNG LAVAGE FLUID*

Control Animals

		4204 (C_1)	4206 (C_2)	4208 (C_3)	4210 (C_4)	4212 (C_5)	4214 (C_6)	4216 (C_7)	4218 (C_8)	4220 (C_9)
Readings	(1)	.08660	.06497	.07914	.10456	.11352	.10000	.09055	.10750	.08203
	(2)	.08615	.05997	.07583	.09700	.11411	.10252	.09635	.11752	.07420
	(3)	.08645	.05989	.07177	.08512	.11347	.11065	.09234	.10408	.08014
	$\bar{M}$	.08640 (±.00022)	.06161 (±.00291)	.07558 (±.00369)	.09556 (±.00979)	.11370 (±.00035)	.10439 (±.00556)	.09308 (±.00296)	.10970 (±.00698)	.07879 (±.00408)

Exposed Animals

		4205 (X_1)	4207 (X_2)	4209 (X_3)	4211 (X_4)	4213 (X_5)	4215 (X_6)	4217 (X_7)	4219 (X_8)	4221 (X_9)
Readings	(1)	.16227	.13744	.11299	.12578	.14814	.15142	.15214	.15558	.07160
	(2)	.15854	.14200	.11866	.13818	.14325	.15620	.15011	.14276	.06917
	(3)	.18865	.14521	.11752	.11968	.14751	.15531	.15108	**	**
	$\bar{M}$	.16982 (±.01641)	.14155 (±.00390)	.11639 (±.00299)	.12788 (±.00942)	.14630 (±.00266)	.15431 (±.00254)	.15111 (±.00101)	.14917 (±.00906)	.07038 (±.00171)

(1) * Expressed in arbitrary units according to weights of gel scan cut-outs (Gm)

(2)** Damaged gel

Studies were also carried out in an attempt to correlate proteinuria and disphosphoglycerate content of red blood cells with the protein content of the lavage fluid. However, the findings were not technically satisfactory and the data were not published.

Summary

An analysis of protein content in lung lavage fluid by PAGE and densitometric measurements has shown a greater amount of protein in the lavage fluid from guinea pigs exposed to an ambient level of NO_2, namely 0.4 ppm continuously for one week ($p < .001$). The loss of protein was considered to reflect an altered capillary permeability from the NO_2 exposure, and the possibility exists that some of the protein may represent an increased turnover of lung cells. The PAGE method developed for this study is a very practical means for detecting this important pathophysiologic alteration. In terms of meaning for air quality standards, it is well recognized that increased protein content of the urine is an important sign of renal impairment. We believe the same principle applies to the lung although we do not presently have such information in respect to human populations and air quality. Further, the animal model system appears to be an excellent means of providing this new kind of discriminant for assistance with the establishment of air quality standards.

0.36 ppm NO_2 continuously for one week

Measurements of diphosphoglycerate content of red blood cells of guinea pigs

There were nine pairs of animals in the experiment, but only four pairs, or eight animals, were investigated for 2, 3-DPG content of their RBC. The specimens of the other animals were unsuitable for analysis due to storage in the refrigerator beyond 48 hours. Quadruplicate blood samples for each animal were tested and showed consistently high levels for those specimens from the NO_2 exposed animals ($p < .05$) (Table 8). The mean values for the control animals ranged from 16.5 µmol to 20.4 µmol, and the standard deviations were less than 1 µmol with one exception. For the exposed animals, the mean values were 21 µmols to 32.2 µmols, with standard deviations from 0.7 µmold to 3.7 mols.

Summary

The 2,3-DPG content of red blood cells of eight guinea pigs, half of which were exposed to 0.4 ppm continuous NO_2 for one week, showed significant differences, with the exposed animals having an increased content ($p < .05$). While the findings are clearly preliminary, the study did demonstrate the feasibility of this newly developed, simplified method for the determination of DPG, and it also showed good reproducibility. There has been no prior report dealing with air pollutant effects on DPG other than mention of its dissociation from hemoglobin under the influence of carbon monoxide in tissue culture preparations. The significance of the finding is that the DPG level essentially reflects poor cell oxygenation.* The implication suggested is that an ambient level of NO_2 exposure may affect oxygen binding by the red blood cell.

(* "dysoxia")

Table 8

2,3-DPG Phosphorus Content of RBC (µmol/ml*) in Guinea Pigs

Group	1	2	3	4
Control	21.30	23.20	18.625	17.650
	19.30	24.40	17.025	16.650
	19.80	17.00	16.625	15.550
	20.05	17.00	17.325	16.250
SD	0.8508	3.9564	0.8655	0.8770
Mean	20.1125	20.4000	17.4000	16.5250
Exposed	24.650	21.175	30.925	20.600
	25.450	28.775	34.625	22.000
	17.950	22.775	32.925	20.700
	25.750	24.575	30.325	20.700
SD	3.6959	3.2756	1.9619	0.6683
Mean	23.4500	24.3250	32.2000	21.0000

*DPG µmol values are equal to one half of the phosphorus values.

Exposure of guinea pigs to 2 ppm NO_2 for one week

An automated determination of ratios of Type 2 pneumocytes to alveolar wall area using an image analyzer

In two earlier studies Type 2 pneumocytes were counted by hand and by an image analyzer, with the latter using one of the hand count measurements as a denominator for a ratio determination. In the present study, the lungs of 24 animals, equally divided into control and exposed groups, were analyzed using microscopic slides from an earlier study (30) and substituting one of the serial sections for the original one used. The purpose of the study was to achieve a count of Type 2 cell pneumocytes based entirely on image analysis rather than be dependent upon a hand count for the denominator, i.e. numbers of alveoli. The denominator selected was the wall area as measured by the Quantimet and as displayed by a phase contrast image for image analyzer detection. The details of the study have been previously reported (6). In brief, a stepping stage of the image analyzer automatically selected six fields in addition to an initial one randomly selected by the investigator. Two sets of five scans each were performed in each field, in accordance with five sizing thresholds, i.e. at threshold 1, all detected LDH positive pneumocytes were counted, including those represented by a small amount of cytoplasm. The other thresholds (2-5) detected cells with diameters greater than 5, 10, 15, and 20 micra respectively. The LDH positive Type 2 pneumocytes were quantitated with light transmission microscopy; the alveolar walls by phase contrast without changing the field. Data analysis was carried out for five values of Type 2 pneumocytes and nine for wall areas, for the lungs of each animal. The 45 sets of ratios were correlated with the ratios of cell/alveoli obtained from the hand count analysis of corresponding photographic slides. The results are presented in Table 9, which shows that of the nine correlations between numbers of alveoli counted by hand and those evaluated by image analyzer, the correlation with $Wall_1$-$Wall_2$ was the most significant ($R = 0.563$; $p < 0.002$). Figure 6 shows the 24 data pairs as a graphic plot. Of the five correlations between the numbers of alveolar wall cells counted by hand and those by image analyzer, the correlation of $cell_1$ was the most significant ($R = 0.77$; $p < 0.001$). However, all other correlations were almost as significant. Table 10 presents the correlations between the ratios of cells/alveoli from the hand count and those from the cell/wall area; these gave the highest correlation coefficient. The conclusion was made that the tedious hand count methodology of cells and alveoli could be abandoned completely in favor of cells and wall area in view of the strong correlation ($R > 0.7$; $p < 0.001$).. Further the methodology has now opened the way for a greatly expanded study not only of numbers of Type 2 pneumocytes but increases in size as well. In addition, a mathematical analysis for determining numbers of alveoli and internal surface area from the image analysis methodology now appears to be feasible.

Summary

A method has been developed which permits a totally automated quantitation of numbers and size of alveolar wall cells and amount of wall area, for the purpose of quantitating hyperplasia and hypertrophy of Type 2 pneumocytes following air pollutant exposure at near ambient and/or ambient levels. The

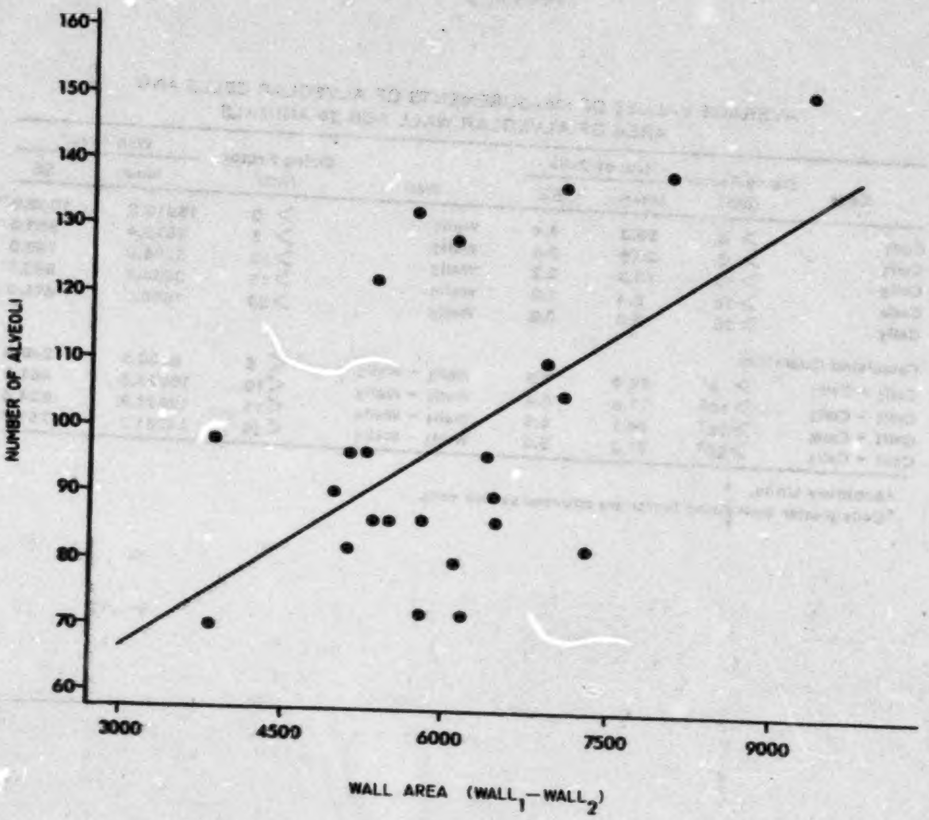

Figure 6. Plot of hand count of alveoli versus automated measurements of wall area for the 24 slides. The correlation coefficient is 0.563.

Table 9

AVERAGE VALUES OF MEASUREMENTS OF ALVEOLAR CELLS AND AREA OF ALVEOLAR WALL FOR 24 ANIMALS

Cells	Sizing Factor (μm)	No. of Cells		Wall	Sizing Factor (μm)	Wall Area*	
		Mean	SE			Mean	SE
Cell$_1$	> 0	58.3	4.4	Wall$_1$	> 0	15910.3	1049.5
Cell$_2$	> 5	30.6	3.6	Wall$_2$	> 5	9879.4	983.0
Cell$_3$	> 10	13.3	2.2	Wall$_3$	> 10	5296.0	785.5
Cell$_4$	> 15	6.4	1.8	Wall$_4$	> 15	3034.4	580.7
Cell$_5$	> 20	3.0	0.8	Wall$_5$	> 20	1658.6	415.0
Calculated Quantities							
Cell$_1$ + Cell$_2$	$> 5^{\dagger}$	89.0	7.9	Wall$_1$ − Wall$_2$	< 5	6030.9	249.9
Cell$_1$ + Cell$_3$	$> 10^{\dagger}$	71.6	6.4	Wall$_1$ − Wall$_3$	< 10	10614.3	451.2
Cell$_1$ + Cell$_4$	$> 15^{\dagger}$	64.7	5.5	Wall$_1$ − Wall$_4$	< 15	12875.8	624.6
Cell$_1$ + Cell$_5$	$> 20^{\dagger}$	61.3	5.0	Wall$_1$ − Wall$_5$	< 20	14251.7	751.7

*Arbitrary Units.

†Cells greater than sizing factor are counted as two cells.

Table 10

COEFFICIENTS OF CORRELATION BETWEEN RATIOS OF CELLS TO ALVEOLI OBTAINED BY HAND COUNT AND AUTOMATED DETERMINATION OF RATIO OF CELLS TO WALL AREA USING SELECTED NUMERATORS AND DENOMINATORS

	Denominator				
Numerator	$Wall_1$	$Wall_1 - Wall_2$	$Wall_1 - Wall_3$	$Wall_1 - Wall_4$	$Wall_1 - Wall_5$
$Cell_1$	0.42	0.79	0.73	0.66	0.58
$Cell_1 + Cell_2$	0.63	0.80	0.79	0.77	0.73
$Cell_1 + Cell_3$	0.64	0.80	0.79	0.77	0.73
$Cell_1 + Cell_4$	0.56	0.79	0.77	0.73	0.68
$Cell_1 + Cell_5$	0.51	0.79	0.76	0.71	0.64

study has demonstrated a high correlation between hand and automated cell counts ($R > 0.7$; $p < 0.001$) and between hand counts of alveoli and automated wall area measurements ($R > 0.56$; $p < 0.002$). A great usefulness of this methodology for assistance in the establishment of air quality standards is indicated by these findings. There is also a great potential for other applications, including assistance in the definition of early pathologic lesions.

0.4 ppm NO_2 exposure, 4 hrs/day, for one week and three weeks

Acid phosphatase in density equilibrium fractions of lungs of guinea pigs

Three independent experiments, each including eight guinea pigs, half exposed to 0.4 ppm NO_2 for four hours/day for seven days, were carried out for acid phosphatase content of lung homogenates centrifuged on a 5%-6% linear sucrose density gradient. Five distinct bands, from six pooled fractions, were observed in each gradient (Table 11) and these were isolated, pooled, and tested spectrophotometrically for acid phosphatase activity. Two differences were found with respect to the NO_2 exposed animals in all of the three studies, a greater overall (all fractions) amount of acid phosphatase activity ($p < .025$) and a density gradient region containing primarily membrane fragments which appeared to contribute most heavily to the overall difference in acid phosphatase activity ($p < .001$). The details are provided in Tables 12-15. The findings are considered to be of a preliminary nature in view of the small number (24) of animals involved in the study, but there is the implication that this ambient level of NO_2 applied intermittently for one week may have resulted in subtle alterations in parenchymal cells and macrophages with a resultant increase of lysosomal enzymes.

Ultrastructure

The studies showed the pooled fraction with the highest content of acid phosphatase to be characterized by membrane fragments and cytoplasmic debris; no specific structural relationship was established, but other fractions had much less of this material.

Summary

In three independent experiments, 24 guinea pigs were exposed to 0.4 ppm NO_2, four hours/day, for seven days (Experiments 1 and 2) and 21 days (Experiment 3). Density gradient centrifugation was carried out with the homogenized lung tissue and acid phosphatase determined in five pooled fractions of each gradient. The acid phosphatase content of all five fractions was higher in the exposed group of animals than in the controls ($p < .025$). There were also significant differences between the pooled fractions ($p < .001$). The acid phosphatase content, as determined by assay of the supernate from whole lung homogenates, did not show differences due to NO_2 exposure.

Continuous 0.5 ppm NO_2 for seven days and 14 days, and intermittent 0.4 ppm NO_2, four hours/day for eight, nine and ten days.
Proteinuria in guinea pigs

Protein quantitation

Table 11. BAND PROPERTIES

Pooled Fractions	Bands	Band Position*	Mean Density of Band	Band Width (mm)	Fractions Pooled	Band Margins
I	Fluffy white	1.0	2.174	2.0	1, 2	Vague
II	Light brown	2.2	2.077	1.0	3, 4, 5	Sharp
III	(No band observed)	---	---	---	6, 7, 13, 14	---
IV	Opaque white	3.9	1.939	3.5	8, 9, 10, 11, 12	Vague
V	Opaque white	4.8	1.866	2.0	15, 16, 17 18	Vague
VI	Translucent light brown	5.2	1.834	1.5	19, 20	Sharp

*Distance (cm) from bottom of gradient tube

Table 12. ACID PHOSPHATASE READINGS*
(Experiment 1)

		Pooled Fraction					
		I	II	III	IV	V	VI
Control Animals	C 1	.0159	.0214	.0194	.0189	.0104	.0085
	C 2	.0101	.0080	.0078	.0106	.0142	.0129
	C 3	.0109	.0213	.0210	.0225	.0113	.0103
	C 4	.0161	.0196	.0204	.0199	.0122	.0121
Mean		.013250	.017575	.017150	.017975	.012025	.010950
SD		.003193	.006436	.006268	.005145	.001625	.001962
Exposed Animals	X 1	.0244	.0217	.0187	.0194	.0102	.0078
	X 2	.0259	.0259	.0232	.0248	.0154	.0099
	X 3	.0093	.0229	.0248	.0249	.0119	.0118
	X 4	.0132	.0236	.0188	.0131	.0174	.0178
		.018206	.023525	.021375	.020550	.013725	.011825
		.008204	.001767	.003100	.005591	.003269	.004305
Significance of Treatment		p < .05	p < .05	p < .05	NS**	NS**	NS**

* Adjusted for protein content of each pooled fraction

** NS = Not significant - p > .05

Overall significance: p < .025

Table 13. ANALYSIS OF VARIANCE OF ACID PHOSPHATASE DATA (Experiment 1)

	Degrees of Freedom	Mean Squares	F Ratio	p Value
Treatment (T)	1	.000137	6.22	< .025
Fraction (F)	5	.000114	5.19	< .001
T x F	5	.000008	0.35	NS
Animal A (TF)	36	.000022		

Table 14. ACID PHOSPHATASE READINGS*

(Experiment 3)

		Pooled Fraction					
		I	II	III	IV	V	VI
Control Animals	C 1	.0030	.0072	.0081	.0126	.0033	.0019
	C 2	.0043	.0089	.0108	.0158	.0038	.0030
	C 3	.0138	.0142	.0161	.0143	.0029	.0022
	C 4	.0013	.0042	.0068	.0089	.0024	.0018
Mean		.005600	.008625	.010450	.012900	.003100	.002225
SD		.005602	.004193	.004118	.002969	.000594	.000543
Mean**		.002933	.006766	.008566	.012433	.003166	.002233
SD**		.001601	.002379	.002040	.003453	.000709	.000665
Exposed Animals	X 1	.0035	.0092	.0110	.0157	.0031	.0019
	X 2	.0041	.0127	.0127	.0169	.0031	.0022
	X 3	.0069	.0107	.0106	.0135	.0034	.0027
	X 4	.0041	.0117	.0087	.0113	.0025	.0047
Mean		.004650	.011075	.010750	.014350	.003025	.002875
SD		.001526	.001493	.001642	.002473	.000377	.001260
Significance of Treatment		NS	$p < .05$**	NS	NS	NS	NS

* Adjusted for protein content of each pooled fraction

** Data based on exclusion of one animal (C 3)

NS = Not significant - $p > .05$

Overall significance: N.S. ($p < .005$ without animal C3)

Table 15. ACID PHOSPHATASE READINGS*
(Experiment 3)

		Pooled Fraction					
		I	II	III	IV	V	VI
Control Animals	C 1	.161	.496	.777	1.140	.848	.576
	C 2	.455	.566	.681	1.088	.718	.596
	C 3	.657	1.121	1.440	.909	.646	.424
	C 4	.061	.221	.566	.676	.520	.421
Mean		.333500	.601000	.866000	.953250	.683000	.489250
SD		.272902	.377292	.392267	.209649	.137098	.083719
Exposed Animals	X 1	.301	.655	.956	1.435	.897	.523
	X 2	.403	.887	1.107	1.577	.903	.585
	X 3	.315	.731	.978	1.163	.905	.630
	X 4	.190	1.226	.986	.862	.588	.851
Mean		.302250	.874750	1.006750	1.259250	.823250	.647250
SD		.087397	.253298	.006750	.315660	.156870	.142740
Significance of Treatment		NS**	NS**	NS**	NS**	NS**	NS**

* Adjusted for protein content of supernate (whole homogenate)

** NS = Not significant - $p > .05$

Overall significance: $p < .025$

This study consists of two independent experiments, one involving a colony of 14 guinea pigs, half of which were exposed to continuous NO_2, with testing of three of the animals at seven days and four at 14 days. 2) In the second experiment, there were 24 animals, half of which were exposed to 0.4 ppm NO_2 for four hours per day, with testing at 8, 9, and 10 days after exposure. The urine was collected from the bladder with a sterile hypodermic needle and syringe and processed for polyacrylamide gel electrophoresis (Fig. 7), Lowry protein determinations, and refractometer measurements of specific gravity. The results have been reported previously (8). In brief, the two-factor analysis of variance statistical study showed significant effects for NO_2 exposure ($p < .01$ for the first experiment and $p < .05$ for the second experiment but without an influence by the duration of exposure, i.e. one week vs. two weeks. The same statistical significance was found in both of the analyses carried out, i.e. PAGE and Lowry protein determinations (Tables 16-20). In a study of water consumption by 162 control and 116 experimental animals in four independent experiments, no statistically significant differences were found.

Ultrastructure and histopathology

Representative sections of the cortex and medulla of the kidney were processed for ultrastructural examination as well as routine histologic study. No kidney abnormality was noted on routine study. The ultrastructural studies are incomplete (loss of Dr. Ted Yuen to the project).

Summary

Urinary proteins were quantitated by PAGE, Lowry protein tests, and refractometry. There were two experiments, with 14 animals in the first with continuous 0.5 ppm exposure for one and two weeks, and 24 in the second with four-hour per day 0.4 ppm NO_2 exposure for eight to ten days. Both PAGE and Lowry protein measurements showed a greater amount of urinary protein for the exposed animals ($p < .01$ and $p < .05$ respectively). While this study is clearly of a preliminary nature, there is an implication that exposure to NO_2 may have an adverse systemic as well as local lung effects. An increase in protein content of the urine is often an early indication of subclinical renal disease in humans and this often occurs in the absence of structural abnormality by routine histological examination.

Continuous 0.47 ppm NO_2 for ten, 12 and 14 days
Protein leakage in the mouse lung

Fluorescamine labeling studies

Forty-four Swiss Webster male mice were divided equally into control and experimental groups, matched according to weight. A fluorescamine solution was injected immediately after its constitution (2 mg of fluorescamine powder in 0.15 ml of 100% ethyl alcohol, followed by a mixing of 0.05 ml of saturated supernate to basal salt solution for a final volume of 0.4 ml. The solution was injected into a tail vein over a period of three minutes ± 30 seconds. At precisely 40 minutes after injection, and following a lethal dose of intraperitoneal pentobarbital sodium, the chest cavity was opened and blood collected from the right ventricle. Fifty µl of serum was placed in 0.5 ml

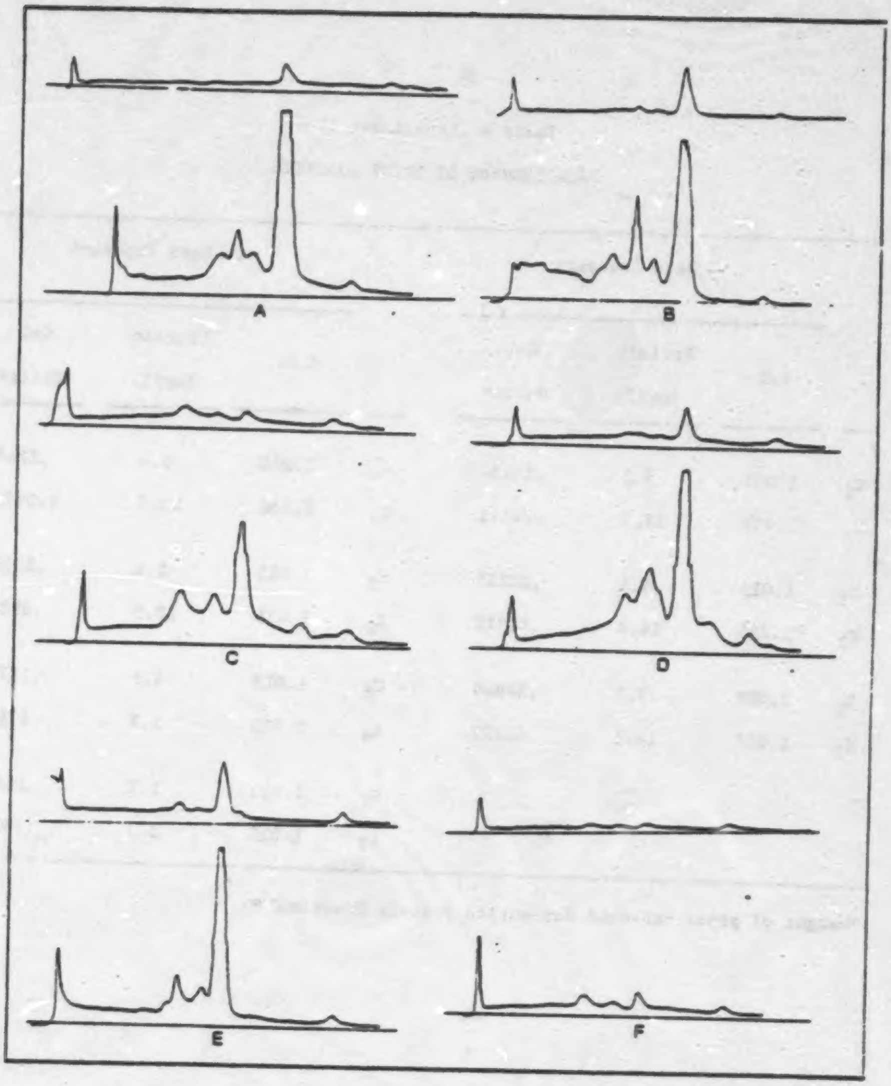

Figure 7. Comparisons of densitometric readings: disc-gel electrophoretic determinations of urinary protein. A representative number of gel scan tracings are displayed, control group on top and exposed on bottom. As a rule, three major peaks presumably albumins, combined α- and β-globulins and γ-globulins (right to left; extreme left peak is tracking dye) were noted, as shown in C, D, E, F. In a few instances, four peaks were found (A and B). Control urine with a very flat curve (F), had a low specific gravity (1.013), but B shows a prominent control urine peak with an even lower value, 1.008.

Table 16. (Experiment 1)

DATA SUMMARY OF URINE ANALYSIS

	7 Days Exposure				14 Days Exposure		
	S.G.	Protein (mg/%)	Gel Units*		S.G.	Protein (mg/%)	Gel Units*
C_1	1.007	5.2	.13134	C_4	1.008	5.4	.22477
X_1	>1.035	17.1	.74471	X_4	1.018	23.7	1.09729
C_2	1.013	5.4	.22255	C_5	1.013	5.4	.23359
X_2	>1.035	14.8	.62972	X_5	1.035	19.5	.89827
C_3	1.009	5.7	.34600	C_6	1.013	4.9	.11765
X_3	1.027	14.8	.62227	X_6	1.020	5.7	.27630
				C_7	1.013	5.2	.14577
				X_7	1.012	5.3	.20001

*Weight of paper cut-outs for entire protein fraction

Table 17. (Experiment 2)

ALBUMIN CONTENT OF URINE DISC-GEL ELECTROPHORESIS

	8 Days Exposure					9 Days Exposure					10 Days Exposure			
	Sample* 1	Sample 2	$\bar{X}$	S.D.		Sample* 1	Sample 2	$\bar{X}$	S.D.		Sample* 1	Sample 2	$\bar{X}$	S.D.
C_1	71	67	69	2.83	C_5	34	40	37	4.24	C_9	301	330	315.5	20.51
X_1	125	140	132.5	10.61	X_5	221	209	215	8.48	X_9	32	32	32	0
C_2	151	157	154	4.24	C_6	52	60	56	5.66	C_{10}	47	48	4.75	.71
X_2	211	223	217	8.49	X_6	31	28	29.5	2.12	X_{10}	52	50	51	1.00
C_3	56	54	55	1.41	C_7	39	39	39	0	C_{11}	400	390	395	7.07
X_3	—	—	—	—	X_7	274	260	267	9.90	X_{11}	532	500	516	22.63
C_4	44	44	44	0	C_8	154	160	157	4.24	C_{12}	306	321	313.5	10.61
X_4	108	96	107	8.48	X_8	347	331	339	11.31	X_{12}	1500	1400	1450	70.71

* Sample units are based on weights of paper cut-outs from albumin fraction.

Table 18
(Experiment 2)

	8 Days Exposure			9 Days Exposure			10 Days Exposure	
	Urine Vol. ml	Urine S.G.		Urine Vol. ml	Urine S.G.		Urine Vol. ml	Urine S.G.
C_1	4.4	1.023	C_5	2.0	> 1.035	C_9	1.0	1.035
X_1	1.4	1.015	X_5	2.4	1.021	x_9	3.6	1.021
C_2	0.5	1.028	C_6	2.0	> 1.035	C_{10}	2.0	1.031
X_2	0.9	1.017	X_6	2.2	1.029	X_{10}	0.8	1.028
C_3	3.8	1.035	C_7	0.4	1.028	C_{11}	2.0	> 1.035
X_3	--	--	X_7	1.0	1.022	X_{11}	2.0	1.021
C_4	1.0	1.035	C_8	2.0	1.035	C_{12}	1.6	> 1.035
X_4	1.0	1.028	X_8	5.0	1.027	X_{12}	3.2	1.033

Table 13 (Experiment 2)

TOTAL PROTEIN CONTENT (DISC-GEL) OF URINE

	8 days	9 days	10 days
Control			
Mean	.09094	.14423	.32136
S.D.	.04135	.08915	.19410
Exposed			
Mean	.169113	.28282	.43248
S. D.	.09048	.20680	.41568

* n = 4 for each group, except for the 8 day exposed group where n = 3

Table 20

Protein Content (mg/Z) With and Without Dialysis

	-	+	Gels
C_1	2.000	.456	.1296
C_2	1.020	.471	.1291
X_1	1.085	.703	.1975
X_3	1.754	.828	.2719

basal salt solution at 4°C for fluormetric assay. The lungs were washed briefly in cold basal salt solution, and pressed between two gauze sponges to remove surface blood and capillary blood content, and homogenized. The fluorescence of each sample was recorded between 410 nm and 700 nm, with the excitation wave length set at 390 nm (emission peak was found to be 480 nm). For each of the various samples, a standard was prepared with a fluorescein stock solution that was diluted as needed to 5×10^{-6}. The peak reading obtained from the standard was used to standardize subsequent readings, both at the beginning and the end of each animal pair unit experiments.

Fluormetric readings were obtained of lung homogenates and serum. The findings for the lung are listed in Table 21, and the details have been presented in a published report (9). In brief, there were consistently greater mean values for the exposed animals for each of the three time periods compared with the control counterparts. Also, the mean value of the combined exposed groups (i.e. for all three periods of exposure) was greater than that of the control counterparts ($p < .025$). The results of the serum tests are given in Table 22; no significant differences were found. Further, a linear regression was carried out comparing serum and lung levels of bound fluorescamine. The correlation coefficient was determined to be 0.18547 and thus not significant.

Ultraviolet microscopy

An ultraviolet microscopy study (FITC excitation filter and Zeiss OGI barrier filter, with epiluminescence) showed small aggregates of amorphous material randomly distributed throughout the lung, plus a generalized background fluorescence of the lung tissue which was greater than that seen with the autofluorescence of lungs from mice not receiving the fluorescamine injection. The overall impression was that labeled protein was distributed throughout all tissues of the lung, with no aggregate localization that was consistent in terms of distribution and amount. It was not possible to distinguish control from exposed animals by microscopy. The findings are consistent with others who have shown a bidirectional transport of protein in the lung, which includes intracellular protein aggregates and lymphatic clearance. This is the first study using the fluorescamine label in an attempt to demonstrate a greater loading of the protein transport system of the lung following air pollutant exposure, and also appears to be the first to attempt this type of quantitation regardless of methodology. Hypothetically, a measure of protein transport in the lung may be much more sensitive than lavage fluid measurements in view of the great capacity of the cells of the lung for protein reabsorption.

Summary

The protein content of homogenized lung tissue from 44 mice, half of which were exposed continuously to 0.47 ppm NO_2 for 10, 12, and 14 days, was assayed fluormetrically following intravenous injection of fluorescamine, a new protein labeling reagent for quantitative assay use. The mean protein values of all exposed animals were higher than those of the control animals ($p < .025$). No correlation was noted between serum fluorphor levels and those in the lung homogenate. Ultraviolet microscopy studies support the working hypothesis that protein leakage is associated with an increased load of protein in the protein transport system of the lung.

TABLE 21

LUNG HOMOGENATE FLUORESCENCE

	DAY 10	DAY 12	DAY 14
CONTROL	79	85	39
	87	79	71
	78	130	89
	92	56	97
	69	102	146
	82	52	150
	48	–	78
	95	–	89
	mean: 78.75 S.D.: 14.92	mean: 84.00 S.D.: 29.23	mean: 94.87 S.D.: 37.23
EXPOSED	72	106	79
	87	81	80
	88	128	102
	107	114	67
	79	120	96
	108	90	205
	119	–	300
	140	–	500
	mean: 100.00 S.D.: 22.71	mean: 106.50 S.D.: 18.01	mean: 178.62 S.D.: 152.83

* Control mean: 86.0
S.D.: 28.1
Exposed mean: 130.4
S.D.: 97.1

$p < .025$

TABLE 22

SERUM FLUORESCENCE

	DAY 10	DAY 12	DAY 14
CONTROL	143	179	76
	169	160	40
	95	87	119
	111	115	72
	132	171	163
	150	146	78
	141	-	-
	-	-	-
	mean:134.42 S.D.: 24.71	mean:143.00 S.D.: 35.49	mean: 91.33 S.D.: 43.18
EXPOSED	152	141	119
	83	132	104
	91	201	125
	86	242	147
	150	209	200
	108	114	178
	75	-	-
	-	-	-
	mean:106.42 S.D.: 32.06	mean:173.16 S.D.: 51.04	mean:145.50 S.D.: 37.07

*		
	Control mean:	123.5
	S.D.:	39.9
	Exposed mean:	139.8
	S.D.:	47.6

$p < .25$

Continuous 2.0 ppm NO_2
An experimental model and automated methodology

Experimental model

The data from an experiment involving 24 white, all male guinea pigs, half of which were exposed to NO_2 for 1, 2, and 3 weeks, were analyzed by a partially nested analysis of the variance model. There were three crossed factors: 1) treatment (T) with two levels (control or NO_2 exposed); 2) duration (D), with three levels (1, 2, or 3 weeks); 3) and lobe (L), with two levels (upper and lower). The other factors in the design were the error factors given for the four animals (A) for each treatment-duration lobe (TDL) combination; the section (S) factor (three levels) nested within treatment-duration lobe animal (ATDL); and count factor (Error) with two levels nested within all factors. The expression for this model system is given in Figure 8. Table 23 presents the results of the analysis of variance. The results show that exposure to NO_2 after 2 ppm level resulted in an increase in $\bar{Y}$ from 1.99 to 2.23 cells/alveolus ($p = .06$). Thus, this experimental model was shown to detect a 12% increase in $\bar{Y}$ using only small numbers of animals and a slightly supra-ambient concentration of NO_2.

Automated methodology

Using the data from the earlier studies on automated counting of cell size and cell numbers, various estimates of X1 and X2 were considered as partially shown in Figure 9. The quantities $cell_1$ and $wall_j$ served as correction factors in the estimation. Since $cell_i$ is a count of cells larger than 5(i -1) µM, and since these cells have already been counted in $cell_1$, the $cell_1$ plus $cell_i$ effectively counts these cells twice. Hence in this way $cell_1$ plus $cell_i$ corrects for the counting of two contiguous cells as one large cell. Similarly, the subtraction of $wall_j$ from $wall_1$ has the effect of excluding walls thicker than 5 (j-1) µM. This is desirable since alveolar walls are very thin. By correlating quantities $\check{X}_1$ and $\check{X}_2*$ (averaged over the seven microscopic fields). With X_1 and X_2 respectively, it was demonstrated that the best estimator of X_1 was $\check{X}_1 = cell_1$ ($r = 0.77$; $p < .001$), and the best estimator of X_2 was $X_2^* = wall_1 - wall_2$ ($r = 0.56$; $p < .002$). Since the 35 mm slides could not be used for a phase contrast study, the area scanned was slightly different, and hence a less pronounced correlation of wall area with alveoli was anticipated. The correlation of $Y^* = \check{X}_1/\check{X}_2*$ with Y was $r = 0.79$ ($p < .001$). For a fuller explanation, see the report (12).

Automated quantitation of hypertrophy

The analysis of variance of the data (using averages for each of the 24 animals) revealed no significant treatment-duration-interaction effect, but a significant treatment effect ($F = 6.93$, $p < .02$), and a significant duration effect ($F = 5.33$, $p < .05$). Although no direct correlation with a hand quantitation was possible, these results closely parallel results of the analysis of the variance of the hand hyperplasia analysis of variance (treatment $p < .06$, duration $p < .025$; Table 23.

The present study adds strong support to the feasibility of the automated approach to lung cell and lung tissue quantitation. In particular, the image

Figure 8
Definition of the Model

$$Y_{ijklmn}=\mu+\tau_i+\delta_j+(\tau\delta)_{ij}+\lambda_k+\alpha(\tau\delta\lambda)_{ijkl}+\varepsilon(\alpha\tau\delta\lambda)_{ijklm}+e_{ijklmn}$$

Figure 9
Estimates of X_1 and X_2^*

$$\tilde{X}_1 = \begin{cases} \text{cell}_1 \\ \text{cell}_1+\text{cell}_i \end{cases}$$

for $i=2,\ldots5$, and

$$X_2^* = \begin{cases} \text{wall}_1 \\ \text{wall}_1-\text{wall}_i \end{cases}$$

Estimates of X_1 and X_2^*

Table 23

THE ANALYSIS OF VARIANCE OF THE EXPERIMENTAL DATA

Source	df	MS	*F*	*P*
T	1	4.02	3.94	.06
D	2	4.69	4.60	<.025
L	1	0.42	0.41	NS
TD	2	3.62	3.54	NS
A(*TDL*)	41	1.02	7.85	<.001
S(*ATDL*)	96	0.13	3.25	<.001
Error	144	0.04	—	—

analysis methodology has the potential of contributing to the definition of early pathologic lesions in the lung, especially with respect to the types of lung damage that result in cell population shifts and the covert loss of the cell populations themselves.

Summary

A series of studies designed to develop and automate a method for the quantitation of Type 2 pneumocyte populations of the lung and their changes in response to 2 ppm NO_2 exposure has been presented and discussed. The validity and sensitivity of the automated approach was strongly supported by high correlations between manual and automated cells counts ($r > 0.8$, $p < 10^{-6}$) and between their respected baselines (numbers of alveoli and alveolar wall area; $r = 0.56$, $p < .002$) as well as by the automated detection of significant hypertrophy of Type 2 cells following NO_2 exposure ($p < .025$).

Horseradish peroxidase studies at three levels of NO_2 exposure: continuous 0.5 ppm NO_2, intermittent 0.8 ppm NO_2, and intermittent 0.6 ppm NO_2
Clearance of exogenous horseradish peroxidase in the lungs of mice

PAGE quantitation of lung homogenates

The combined studies resulted in data from 264 mice, with 48 pairs of mice per each experiment except for Experiment 1 (Table 24). The results of this interrelated study of three independently carried out investigations are as follow: 1) the total HRP content (combined α and β bands) of homogenized mouse lung tissue was greater for the exposed animals of Experiments 1 and 2 after three weeks of NO_2 exposure, but not at six weeks of exposure (Tables 25-28); $p < .05$ in both studies by a Student's t test of the three week exposure period alone; 2) in the third experiment, the HRP content of the lung homogenates was again greater for the exposed animals (Tables 29+30) but at six weeks and not at three weeks ($p < .025$); 3) differences in HRP content were also found with respect to the individual α and β bands of HRP, but the findings did not show a consistent pattern for the increases; 4) the serum content of HRP did not appear to be influenced by NO_2 exposure (Table 31); 5) the HRP content of the kidney was increased ($p < .05$) for the exposed group of animals of one of the three experiments (Table 32).

Enzyme kinetic assay

In Experiment 1, the mean HRP lung content at three weeks for exposed animals was again great ($p < .025$; Table 25). For Experiment 2, the three week levels for the exposed animals were greater than those of controls but not significant in view of high variation in the exposed group (6.4 ± 5 control vs. 9.4 ± 11 exposed). In Experiment 3, kidney HRP at six weeks was again greater for exposed animals ($p < .05$; Table 32).

Electron microscopic studies

These were incomplete at the time the project was terminated. A search was made for differences between the control and exposed groups of animals in terms of the presence of HRP in the various types of cell junctions. At the time of termination of the project, no overt differences could be identified.

Table 24a
EXPERIMENTS AND CONDITIONS

Experiment		No.	Duration (Wks)	NO_2 ppm	Exposure Period
1	A	48	3	0.5	Contin.
	B	24	6		
2	A	48	3	0.8	6Hr/day 5 d/wk
	B	48	6		
3	A	48	3	0.6	6Hr/day 5 d/wk
	B	48	6		

Table 24b
MEAN ANIMAL WEIGHTS (GM)

		M108	M109	M111
0 Weeks	C	22.6 ± 2.7	26.8 ± 2.9	29.0 ± 2.0
	X	22.3 ± 1.7	26.5 ± 4.4	29.0 ± 1.9
3 Weeks	C	31.3 ± 2.8	33.4 ± 3.7	35.7 ± 2.2
	X	32.4 ± 3.3	34.0 ± 4.2	36.3 ± 2.6
6 Weeks	C	38.7 ± 3.7	34.2* ± 4.5	38.4 ± 3.1
	X	38.8 ± 3.5	33.3* ± 6.3	39.3 ± 3.3

C: Control Animals

X: Exposed (NO_2) Animals

* 3% mean weight loss in 3 weeks for exposed group and 3% gain for controls vs. 11% → 32% weight gain for M108 and M111 animals at 5 weeks.

TABLE 25. EXPERIMENT 1[†]

HRP CONTENT OF MOUSE LUNG TISSUE (HRP/GM TISSUE)

		3 Weeks		6 Weeks	
Peak	Group	$\bar{M}$	p Value	$\bar{M}$	p Value
α	C	30.53 ± 11.1	< 0.025	44.0 ± 11	N.S.
	X	38.09 ± 12.9		48.4 ± 15	
β	C	8.49 ± 4.0	< .05	9.9 ± 4	N.S.
	X	11.13 ± 5.6	(.1)	12.9 ± 7	
α+β	C	39.02 ± 14.1	< .05	53.8 ± 13	N.S.
	X	49.22 ± 17.8		61.3 ± 22	
α/β	C	3.9 ± 1.2	N.S.	2.7 ± 1.0	< .1
	X	3.8 ± 1.2		2.3 ± 0.8	
HRP/$_{ek}$*	C	10 ± 4	< .025	8 ± 7	N.S.
	X	15 ± 9		12 ± 15	

Values in parentheses are protein compensated and shown only when different

*Enzyme kinetic assay: 10^{-8} Gm HRP/Gm tissue

[†] HRP values by integrated gel scan areas (Peroxidase stained gels)

TABLE 26. EXPERIMENT 1

TWO FACTOR ANALYSIS OF VARIANCE

A. Serum HRP Content*

Peak	NO_2 Factor	Time Factor	NO_2 Time Interaction
α	N.S.	~ 0	N.S.
β	N.S.	~ 0	N.S.
α+β	N.S.	~ 0	N.S.
α/β	< .025	~ 0	N.S.

B. Lung HRP Content*

Peak	NO_2 Factor	Time Factor	NO_2 Time Interaction
α	N.S. (<0.025)	~ 0	N.S.
β	<0.05 (N.S.)	N.S. (~ 0)	N.S.
α+β	<0.05 (<.025)	<.005 (~ 0)	N.S.
α/β	N.S.	< .025	N.S.

*HRP/Gm Tissue

Values in parentheses are protein compensated and shown only when different

TABLE 27. EXPERIMENT 2

HRP CONTENT OF MOUSE LUNG TISSUE (HRP/GM TISSUE)

		3 Weeks		6 Weeks	
Peak	Group	$\bar{M}$	p Value	$\bar{M}$	p Value
α	C	48.4 ± 16	< .1 (N.S.)	48.6 ± 13	N.S.
	X	57.4 ± 22		45.6 ± 10	
β	C	20.9 ± 12	< .05 (N.S.)	10.9 ± 5	N.S.
	X	31.6 ± 32		12.0 ± 8	
α+β	C	69.3 ± 26	< .05 (N.S.)	59.5 ± 16	N.S.
	X	89.0 ± 49		57.7 ± 16	
α/β	C	2.7 ± 1	< .1	5.0 ± 2	< .1
	X	2.3 ± 1		4.3 ± 1	
HRP/ek*	C	6.4 ± 5	N.S.	5.0 ± 2	N.S.
	X	9.4 ± 11		4.9 ± 2	

Values in parentheses are protein compensated and shown only when different

*Enzyme kinetic assay : 10^{-8} Gm HRP/Gm tissue.

TABLE 28. EXPERIMENT 2

TWO FACTOR ANALYSIS OF VARIANCE

A. Serum HRP Content*

Peak	NO_2 Factor	Time Factor	NO_2 Time Interaction
α	N.S.	N.S.	∿ .05
β	< .05	< .001	< .05
α+β	N.S.	< .025	< .05
α/β	N.S.	∿ 0	N.S.

B. Lung HRP Content*

Peak	NO_2 Factor	Time Factor	NO_2 Time Interaction
α	N.S.	N.S. (N.S.)	N.S.
β	N.S.	<.001 (∿ 0)	N.S.
α+β	N.S.	<.005	N.S.
α/β	N.S.	∿ 0	N.S.

Values in parentheses are protein compensated and shown only when different

*HRP/Gm Tissue

TABLE 29. EXPERIMENT 3

HRP CONTENT OF MOUSE LUNG TISSUE (HRP/GM TISSUE)

		3 Weeks		6 Weeks	
Peak	Group	$\bar{M}$	p Value	$\bar{M}$	p Value
α	C	51.2 ± 16.8	N.S.	43.3 ± 13.0	.005 (< .025)
	X	51.6 ± 21.6		59.7 ± 23.7	
β	C	12.5 ± 10.9	N.S.	10.8 ± 6.4	.005 (< .01)
	X	18.2 ± 23.0		23.9 ± 20.0	
α+β	C	63.7 ± 25.6	N.S.	54.1 ± 17.3	.0025 (<.005)
	X	69.8 ± 43.3		83.7 ± 39.8	
α/β	C	5.55± 2.64	N.S.	4.56± 1.37	< .05
	X	5.71± 3.75		3.67± 1.85	

Values in parentheses are protein compensated and shown only when different

TABLE 30: EXPERIMENT 3

TWO FACTOR ANALYSIS OF VARIANCE

Lung HRP Content

Peak	NO_2 Factor	Time Factor	NO_2 Time Interaction
α	< 0.05 (N.S.)	N.S.	< 0.05 (N.S.)
β	0.01 (< 0.025)	N.S.	N.S.
α+β	< 0.025 (< 0.05)	N.S. (< 0.05)	N.S.
α/β	N.S.	< 0.01	N.S.

Values in parentheses are protein compensated and shown only when different

TABLE 31: SERUM 3 WEEKS

		Experiment 1			Experiment 2			Experiment 3		
Peak	Group	$\bar{M}$		p Value	$\bar{M}$		p Value	$\bar{M}$		p Value
α	C	2.9	2.1	< .05	3.4	3	< .01	2.8	2.7	N.S.
	X	3.2	3.0		6.3	5		2.8	3.3	
β	C	1.3	1.0	N.S.	3.0	2	< .005	1.5	1.4	N.S.
	X	1.6	1.2		5.5	4		1.5	1.7	
α+β	C	3.3	3.1	< .1	6.5	6	< .005	4.3	4.1	N.S.
	X	4.8	4.1		11.8	8		4.3	4.7	
α/β	C	1.3	0.3	<.0025	1.1	.4	N.S.	1.9	0.8	N.S.
	X	1.8	0.7		1.0	.3		2.0	0.6	

6 weeks data: No significance for all items of the three experiments.

TABLE 32. HRP CONTENT OF MOUSE KIDNEY TISSUE (HRP/GM TISSUE)

Peak	Group	Experiment 1 $\bar{M}$	Experiment 1 p Value	Experiment 2 $\bar{M}$	Experiment 2 p Value	Experiment 3 $\bar{M}$	Experiment 3 p Value
α	C	29.0±6.0	N.S.	27.0± 6.0	< .1 (< .05)	19.5± 6.0	N.S.
	X	28.0±5.0		31.1±11.0		17.4± 4.0	
β	C	16.0±3.0	< .025 (<.05)	17.0± 7.0	< .025	14.8± 5.0	N.S.
	X	14.0±3.0		25.0±15.0		13.8± 5.0	
α/β	C	1.8±0.3	< .05	1.7± 0.4	< .0025	1.3± 0.3	N.S.
	X	2.1±0.5		1.4± 0.3		1.3± 0.2	
α+β	C	45.0±8.0	N.S.	44.0±12.0	< .05 (<.025)	35.2±10.0	N.S.
	X	42.0±6.0		57.0±26.0		31.3± 9.0	
HRP/$_{pk}$*	C	6.4±1.0	N.S.	5.0± 1.0	< .05		
	X	6.2±1.0		8.0± 7.0			

Values in parentheses are protein compensated, and shown only when different.

*Enzyme kinetic assay: 10^{-8} Gm HRP/Gm tissue.

An evaluation was also started on the relative distribution of HRP in the various cell and tissue compartments of the lung parenchyma.

Summary

In three independent experiments, involving a total of 264 mice, the exogenous HRP content of lungs, kidneys, and serum were determined with and without the influence of three different NO_2 exposures (continuous 0.5 ppm, intermittent 0.6 ppm, and intermittent 0.8 ppm). The purpose of the study was to evaluate possible alterations in capillary permeability and clearance of leaked protein from the lung tissues and alveolar spaces following ambient levels of NO_2 exposure. Both PAGE with gel scanning densitometric measurements and enzyme kinetic assay were carried out to quantitate HRP content in the serum and tissues. In all three experiments, the HRP lung content was greater for the exposed animal groups: 49 ± 18 vs. 39 ± 14 ($p < .05$; 89 ± 49 vs. 69 ± 26 ($p < .05$) and 84 ± 40 vs. 54 ± 17 ($p < .025$). The increase in HRP was found at three weeks for the first two experiments and at six weeks for the third. The findings are believed to represent increased capillary permeability and decreased clearance of HRP from cells, tissues, and spaces of the lung. One of the kidney studies showed an increase ($p < .05$) in HRP content for the exposed animals. No significant differences were noted in the serum content of HRP between the control and exposed groups. Ultrastructural correlations are incomplete.

A potential use of HRP assay for assistance in establishing air quality standards is suggested by the findings. Protein leakage is an important pathophysiologic abnormality and is a common denominator for a great many pathologic lesions at their early stage of development. Delayed HRP clearance tests supplement those for measuring protein leakage into alveolar spaces, and it has the potential for greater sensitivity since the total protein content of the cellular and tissue protein transport system should be several magnitudes greater than the protein content of the alveolar spaces in the early stages of protein leakage.

0.5 continuous and intermittent NO_2 exposures
investigations supported in part by this project

Liquid scintillation counting of labeled cells in culture

One of the exploratory investigations, using tritium labeling methodologies, was applied to material derived from an independently support grant project. Supplemental tissue culture preparations were set up for this specific application. The experiment consisted of a study of 30 mice, half exposed to NO_2 for 36 days, and explantation of lung tissues into culture immediately (0 hours) and after specified periods (5 days and 7 days) of storage in the refrigerator at 4°C ("postmortem interval stress" testing PMI), which we innovated as a means of evaluating vigor (survival time in culture; a separate study) as well as viability of lung cells following *in vivo* NO_2 exposure. For the 0 hour group, 150 cultures with five explants each were pulsed at seven days in vitro with 20 μCi/ml 3H-fucose for 24 hours. The methanol fixed monolayers were evaluated by liquid scintillation counting. The same procedure was carried out for 108 cultures (five-day group) and for 72 cultures (seven-day groups), the decreasing numbers of cultures available reflecting PMI attrition.

The numbers of large, medium, and small colonies found in those cultures which were explanted at 0 hours were as follows: for the control group, 63, 172, and 103 respectively; for the exposed group, 128, 142, and 70 respectively. With respect to the PMI studies, the colonies were measured by a micrometer and separated into six groups, beginning with 1 mm diameters and extending to the largest group of 6 mm in diameter by 1 mm increments. The findings were as follows: at the five day PMI period, the control cultures had 36, 76, 62, 58, 27, and 2 colonies in the respective small to large groups; the exposed had 30, 42, 53, 71, 38, and 12 for the comparable groups. Fo the seven-day PMI period, the findings were: for the controls 18, 31, 30, 58, 11, and 4; for the exposed 12, 26, 52, 53, 24, and 4.

For the 3H-fucose label scintillation counts, there were the following findings: of 150 0 hour cultures, the mean values for the controls and exposed groups were 1039 ± 590 and 1018 ± 462 respectively; these differences were not statistically significant. For the 108 cultures of the five-day PMI group, the control and exposed animals had mean values of 724 ± 400 and 1057 ± 519 respectively, a difference statistically significant ($p < .001$) indicating an increase in numbers of cells, possibly with increased fucose incorporation. For the seven-day groups, there were the following control vs. exposed mean values: 800 ± 401 vs. 779 ± 465. The difference was not statistically significant, but the numbers of cultures were considered to be suboptimal for an appropriate quantitation. In effect, this approach can be considered a stress test of different lung cell populations in a search for discriminants that can quantitate loss of functional and structural reserves. These findings have been presented at the 1976 Federation Meetings (10). ^{3}H-Leucine labeling studies were also done.

Macrophage congregation in tissue cultures of mouse lung

A relatively small part of the study was supported by funds under EPA R 80081-05, primarily assistance in the NO_2 monitoring and other aspects of the in vivo part of the experiment. This work has been published with acknowledgment of R80081 support (11,15). In view of the relatively small role played by this grant support, only a summary of the findings is presented here.

The lungs of 12 mice, half of which were exposed to continuous 0.5 ppm nitrogen dioxide for 3 weeks, were explanted in culture, and the instances of macrophage congregation were quantitated according to numbers of target cells involved, categories of congregation from three to 11 or more, numbers of macrophages participating in each category for the total cultures, and the influence of delaying explantation for 24 and 96 hours. A total of 9042 macrophages and 2140 epithelial and spindle target cells were counted in the outgrowths from 306 explants. The incidence of macrophage congregation (or numbers of target cells) was greater for the cultures from the NO_2 exposed animals, both with respect to total incidences between groups ($p \to 0$), and the 0 hour ($p < 0.001$) and 24 hours ($p < 0.01$) culture sub-groups. In addition, the values for T_3 to T_6 macrophage congregation were individually and consistently greater for the exposed animal group. Postmortem interval stress at 96 hours appeared to result in large colonies, but they were reduced greatly in number. Also the incidence of macrophage congregation fell by 28% as compared to 0 hours and 24 hour subgroups.

Ultrastructural, histochemical aspects

The macrophage congregation study was subsequently extended into ultrastructural, histochemical aspects where, again, some support was provided by EPA-R-80081. A summary of the findings is as follows;

The study is based on mouse lung in tissue cultures, with 85 colonies from control and 104 from exposed animal groups, randomly selected from 626 culture preparations. A quantitative study was made of tritiated thymidine uptake by macrophages in culture with respect to: a) macrophages unassociated with lung cells; b) macrophages associated with lung cells; and c) spindle and epithelial cells. A semi-quantitative study measured the degree of positivity of macrophages for LDH, G6PD, MOH, and ATPase. Those were correlated with post-mortem intervals (PMI), i.e., the explantation after storage at 4°C for 24 hours to 5 days, and with cytostructure, including ultrastructural histochemistry (13,14).

The preliminary findings are: 1) a special macrophage type has been identified. The cell shape is generally round as is the nucleus. The nucleus shows a scalloped chromatin margination, mitochondria are peripherally distributed, dense bodies rare, and the Golgi large; 2) ATPase is strongly positive and LDH weak, while the reverse is true for other macrophages. MDH is also strongly positive whereas only 10% of other macrophages are positive. All are weak with G6PD, but 25% of others do show positivity. The numbers of LDH positive macrophages and the degree of response are greater for the NO_2 exposed mice, but only in the 5 day PMI group ($p < .005$). MDH is also greater at 0 hours ($p < .05$). ATPase is greater for the exposed group for 0 hours, 24 hours, and 96 hours ($p < .01$). ^{3}H-TdR shows no differences between groups (1495 of 4962 exposed vs. 745/2777 for controls, 30% vs. 27%). The uptake for lung cells is greater for the exposed group, $\bar{M} = 45\%$ vs. $\bar{M} = 31\%$ ($p < .05$); 3) at five days PMI, the numbers of macrophages in exposed animal cultures are greater than that of the controls ($p < .025$), based on colony area evaluations.

A definitive publication covering the ongoing (804545) ultrastructural aspects of this work is being prepared for publication (13).

Studies presently incomplete

A brief summary is provided here for those studies which remain incomplete, i.e. 1) studies without definitive results primarily due to technical problems; 2) exploratory studies without definitive results; 3) studies with definitive results, but incomplete at this time due to loss of continuing support for the postdoctoral fellow.

With respect to Dr. Charles Hanson (work period Feb. 1973 to March 31, 1974) five experiments with guinea pigs and one with mice were carried out. They involved the following: 1) 24 guinea pigs exposed to continuous 0.2 ppm for 4 days, 7 days, and 14 days, with studies of lung lavage fluid, serum and urine by PAGE protein quantitation; 2) 51 guinea pigs, with half exposed to intermittent (3 hours per day) 0.8 ppm NO_2 for 3 days, 7 days, 14 days, 21 days, 28 days, and 35 days for PAGE protein content of lung lavage fluid, serum and urine; 3) a study of 32 guinea pigs, half exposed to intermittent (3 hours per day for 3 days) 0.3 ppm with testing of lung lavage protein, plasma, and urine at 0 hours, 3 days, and 6 days postexposure, using PAGE and Lowry protein quantitation; 4) 30 guinea pigs, half of which were exposed to intermittent (4 hours per day) .35 ppm NO_2 for protein and AcPase content of lung lavage fluid and plasma, plus diphosphoglycerate content of red blood cells; 5) 36 guinea pigs, half of which were exposed to continuous 0.42 ppm NO_2 for 7 days, 9 days, 13 days, 15 days, and 4 weeks for a study of blood diphosphoglycerate content, AcPase of lung, and a study of membrane protein of lung and kidney. Dr. Hanson's studies with lung lavage protein determinations confirmed our earlier findings, i.e. an approximately 20-30% increase in protein in the lung lavage fluid from the exposed animals ($p < .05$). His findings with diphosphoglycerate of red blood cells are inconclusive. His work also indicated that density gradient separation appeared to provide positive correlations between exposure and acid phosphatase increase when determinations using whole lung homogenates failed to do so, and the lung homogenate pellet from ultracentrifugation was shown to contain dense aggregates of cell membranes. He was able to demonstrate 18 to 20 bands in his attempt to obtain more specific AcPase fractions; the the results of this work presently remain inconclusive; 6) some studies were carried out by Dr. Hanson with mice exposed to continuous 0.44 ppm. These studies were also exploratory and no conclusive findings were derived from them. The pilot studies were carried out with mouse tissues (lung, kidney, heart, and adrenal gland) as well as with guinea pig tissues, and these studies included investigations of catecholamines.

In an attempt to complete earlier studies carried out by Dr. Ted Yuen, Dr. Yuen was hired on a part-time basis, but could only contribute a relatively small number of hours to the project. Ultrastructural investigations of the transport of ferritin and horseradish peroxidase in the lungs and kidney was studied, in conjunction with the pathobiologic investigation of the main project. An attempt to achieve qualitative and/or quantitative distinctions between tracer molecule distributions in the lungs of control and NO_2 exposed animals at ambient levels of exposure are incomplete at this time. Our working arrangement with Dr. Yuen ended as of December 26, 1976.

Dr. Richters carried out a study involving electronmicroscopy of ferritin deposition in the lungs of 38 animals, half of which had been exposed to 0.5 NO_2. No conclusive differences were found in the distribution between control and exposed animals, but note was made of five exposed animals with unusual sites of deposition as opposed to two control animals.

REFERENCES

1. Sherwin, R.P., Yuen, T.G.H., and Richters, V.: The spontaneous occurrence of wall-free organisms in the lungs of guinea pigs and their association with microvilli and cilia, Am. J. Clin. Path. 60:268-280, 1973

2. Sherwin, R.P., Margolick, J.B., and Azen, S.P.: Hypertrophy of alveolar wall cells secondary to an air pollutant. A semi-automated quantitation. Arch. Environ Health 26:297-299, 1973

3. Margolick, J.B., Azen, S.P., and Sherwin, R.P.: An image analyzer quantitation of type 2 pneumocytes. Am. Rev. Respir. Dis. 108:704-707, 1973

4. Sherwin, R.P., and Carlson, D.A.: Protein content of lung lavage fluid of guinea pigs exposed to 0.4 ppm nitrogen dioxide. Disc-gel electrophoresis for amount and types. Arch. Environ. Health 27:90-94, 1973

5. Mersch, J., Dyce, B.J., Haverback, B.J., and Sherwin, R.P.: Diphosphoglycerate content of red blood cells. Measurements in guinea pigs exposed to 0.4 ppm nitrogen dioxide. Arch. Environ. Health 27:94-95, 1973

6. Sherwin, R.P., Margolick, J.B., and Azen, S.P.:An automated determination of ratios of type 2 pneumocytes to alveolar wall area using an image analyzer Am. Rev. Respir. Disease 108:1015-1018, 1973

7. Sherwin, R.P., Margolick, J.B., and Aguilar, E.A.: Acid phosphatase in density equilibrium fractions of the lungs of guinea pigs exposed intermittently to 0.4 ppm nitrogen dioxide. Fed. Proc. 33:633, 1974

8. Sherwin, R.P., and Layfield, L.J.: Proteinuria in guinea pigs exposed to 0.5 ppm nitrogen dioxide. Arch. Environ. Health 28:335-341, 1974

9. Sherwin, R.P., and Layfield, L.J.: Protein leakage in the lungs of mice exposed to 0.5 ppm nitrogen dioxide. A fluorescence assay for protein. Arch. Environ. Health 31:50-52, 1976

10. Sherwin, R.P., and Richters, V.: The influence of 0.4 ppm nitrogen dioxide in vivo on the outgrowth of lung cells in tissue culture. Fed. Proc. 35:632, 1976

11. Richters, V., Elliott, G., and Sherwin, R.P.: Macrophage congregation in tissue cultures of mouse lungs: Influence of 0.5 ppm nitrogen dioxide in vivo exposure. Fed. Proc. 35:717, 1976

12. Azen, S., Margolick, J.B., and Sherwin, R.P.: An experimental model and automated methodology for the analysis of the effects of ambient levels of air pollution on the lung. Applied Math. Computation 3:95-102, 1977

13. Sherwin, R.P., Richters, V., Bernett, J., and Paller, M.: Macrophage congregation and influence of nitrogen dioxide: Ultrastructural and histochemical aspects. XI Cong. Int. Acad. Path. (accepted for publication)

14. Richters, V., Sherwin, R.P., Paller, M., and Bernett, J.: A multidiscipline postmortem study of the mouse lung macrophage: Influence of 0.6 ppm nitrogen dioxide in vivo. (Presented at the 16th Annual Hanford Biology Symposium, Richland, Washington, September 27, 1976)

15. Richters, V., Elliott, G., and Sherwin, R.P.: Influence of 0.5 ppm nitrogen dioxide exposure of mice on macrophage congregation in the lungs. In Vitro 14:458-464, 1978

16. Sherwin, R.P., Okimoto, D., and Mundy, D.: Sequestration of exogenous peroxidase in the lungs of animals exposed to 0.5 ppm nitrogen dioxide. Fed. Proc. 36:1091, 1977

17. Sherwin, R.P., Margolick, J.B., Aguilar, E.A., and Hanson, C.W.: Intermittent 0.4 ppm nitrogen dioxide exposure of guinea pigs: increase in acid phosphatase in density equilibrium fractions of lung homogenates. (submitted for publication)

18. Sherwin, R.P., Okimoto, D.T., and Mundy, D.I.: Serum and lung clearance of exogenous horseradish peroxidase: Influence of low levels of nitrogen dioxide. (Submitted for publication)

19. Sherwin, R.P., and Yuen, T.G.H: Silicone fluid for the metering and monitoring of nitrogen dioxide. Arch. Environ. Health 24:331-336, 1972

20. Sherwin, R.P., Winnick, S., and Buckley, R.D.: Response of lactic acid dehydrogenase positive alveolar cells in the lungs of guinea pigs exposed to NO_2. Am. Rev. Respir. Dis. 96:319-323, 1967

21. Ornstein, L.: Disc electrophoresis: 1. Background and theory. Ann. NY Acad. Sci. 121:321-349, 1964

22. Lowry, O.H., et al.: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265, 1951

23. cf. Pesce,A.J. Methods Used for the Analysis of Proteins in the Urine Nephron 13: 93-104, 1974,for general review.

24. Yuen, T.G.H., and Sherwin, R.P.: Hyperplasia of type 2 pneumocytes and nitrogen dioxide (10 ppm) exposure: A quantitation based on electron photomicrographs. Arch. Environ. Health 22:178-188, 1971

25. Steinman, R.M., and Cohn, Z.A.: The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. J. Cell Biol. 55:186, 1972

26. Dyce, B.J.: A simple sensitive rabbit assay procedure for 2,3-diphosphoglycerate in multiple specimens of blood. Thesis. University of Southern California, Los Angeles, 1971

27. The Colorimetric Determination of Acid Phosphatase. In Sigma Technical Bull. #104, Aug. 1971

28. Farquhar, M.G., Wissig, S.L., and Palade, G.E.: Glomerular permeability. 1. Ferritin transfer across the normal glomerular capillary wall. J. Exp. Med. 113:47-66, 1961

29. Yuen, T.G.H., Richters, A., and Sherwin, R.P.: Unpublished data

30. Sherwin, R.P., Dibble, J., Weiner, J.: Alveolar wall cells of the guinea pig: increase in response to 2 ppm nitrogen dioxide. Arch. Environ. Health 24:43-47, 1972